NATURE INSIGHT: CARDIOVASCULAR DISEASE

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Insight: Cardiovascular disease

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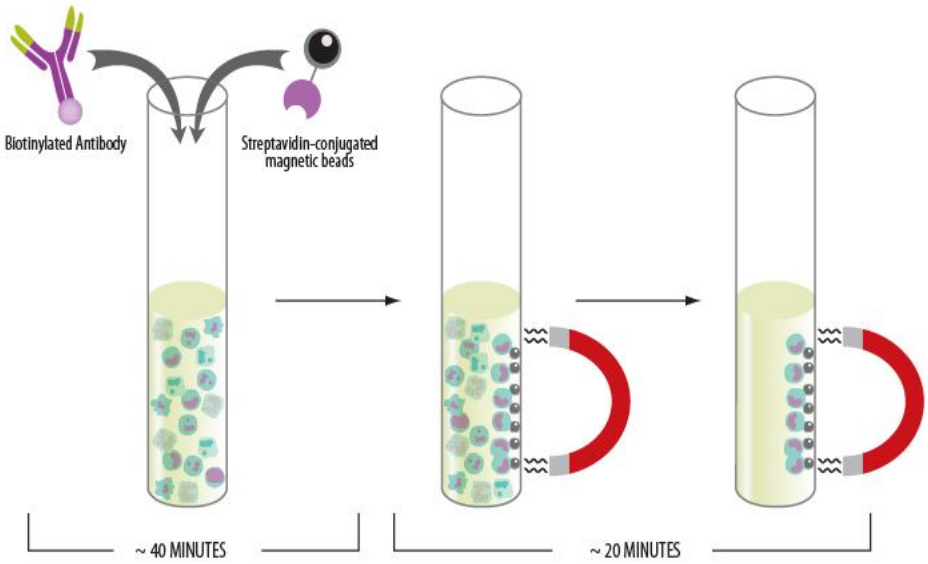

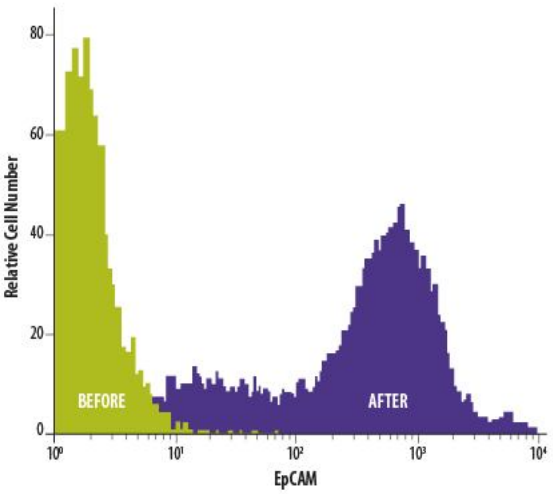

The human EpCAM PlusCellect Kit (Catalog # PLS960) was used to enrich for EpCAM+ cells in a culture containing human whole blood cells spiked with MCF-7 human breast cancer cells. Cells were stained with PE-conjugated anti-human EpCAM detection antibody both before (green) and after (purple) selection.

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Dr. Steven A. Williams
Clark Science Center, Smith College
Northampton, MA 01063

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We are pleased to announce the twenty-third annual Molecular Biology Summer Workshops, sponsored by New England Biolabs in conjunction with Smith College. Workshops are held at the Clark Science Center, Smith College, Northampton, MA, USA. Well over 3,000 people have graduated from this intensive training program in the past twenty-two years.

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No previous experience in molecular biology is required or expected. Fifty participants per session will be selected from a variety of disciplines and academic backgrounds, including principal investigators, directors of programs, medical doctors, postdoctoral fellows, graduate students, research assistants, sales associates, equipment engineers, etc.

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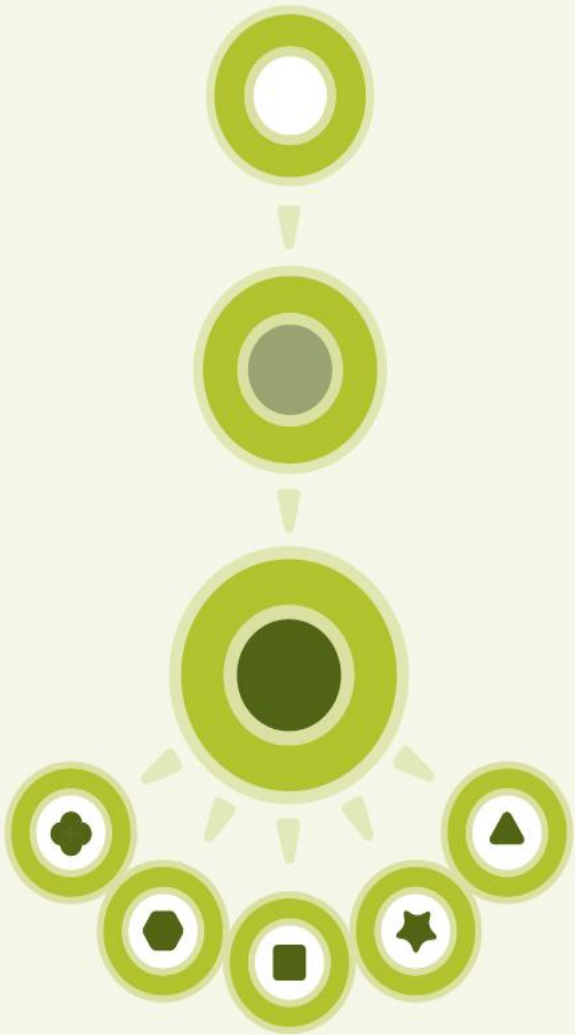

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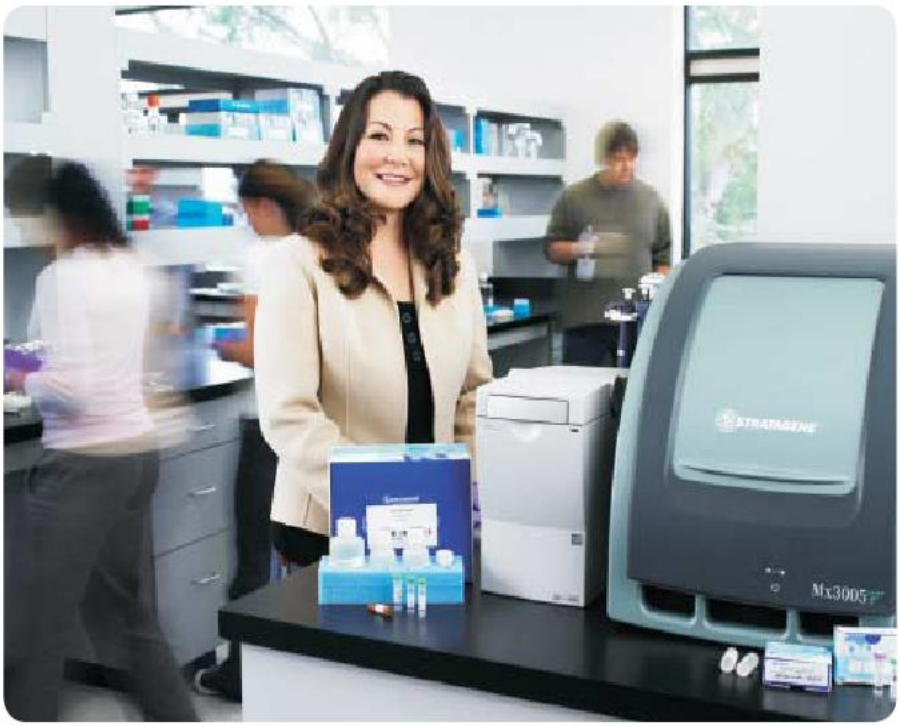

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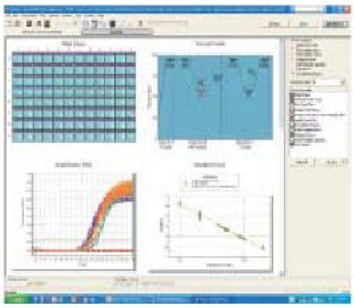

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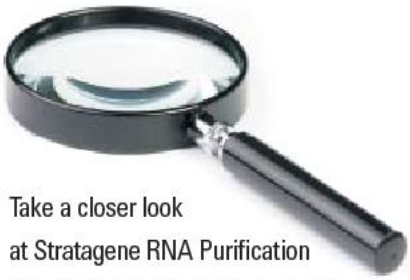

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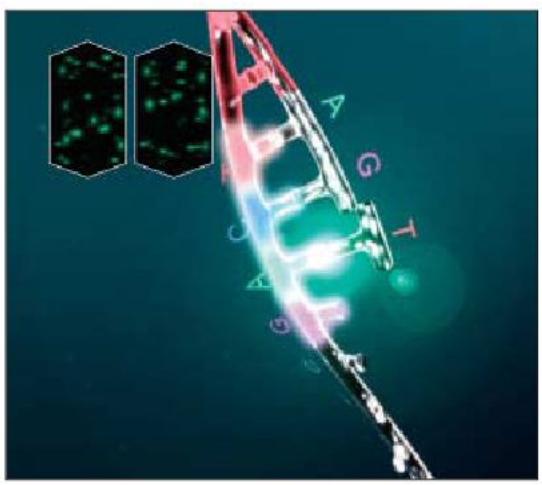

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NEWS

NEWS FEATURES

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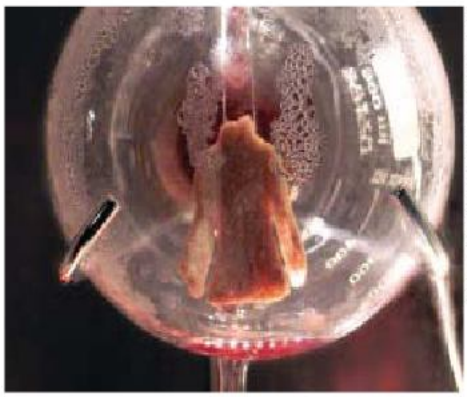
BOOKS & ARTS

US NAVY PHOTOS

Friendly fire: star wars technology could get an airing. News, p. **870**.



VICTIMLESS LEATHER ORON CATTS & IONAT ZURR

Art in tissue culture, p. **891**.

NEWS & VIEWS

NATUREJOBS

FUTURES

My Tetra Is . . . Leakproof

The Mini-Protean® Tetra cell winged locking mechanism locks out leaks.

The Mini-PROTEAN Tetra systems for mini vertical gel electrophoresis feature an innovative locking mechanism that eliminates leakage issues commonly associated with gel electrophoresis. The patented* design makes it easy to lock handcast or precast gels into the electrophoresis module, ensuring leakproof operation and accurate experimental data. Designed to run as many as four SDS-PAGE gels simultaneously, the Mini-PROTEAN Tetra systems offer high throughput and a unique design to meet all your electrophoresis needs.

Key Features

- Patented locking system to eliminate leaks
- Capacity to run up to 4 mini SDS-PAGE gels
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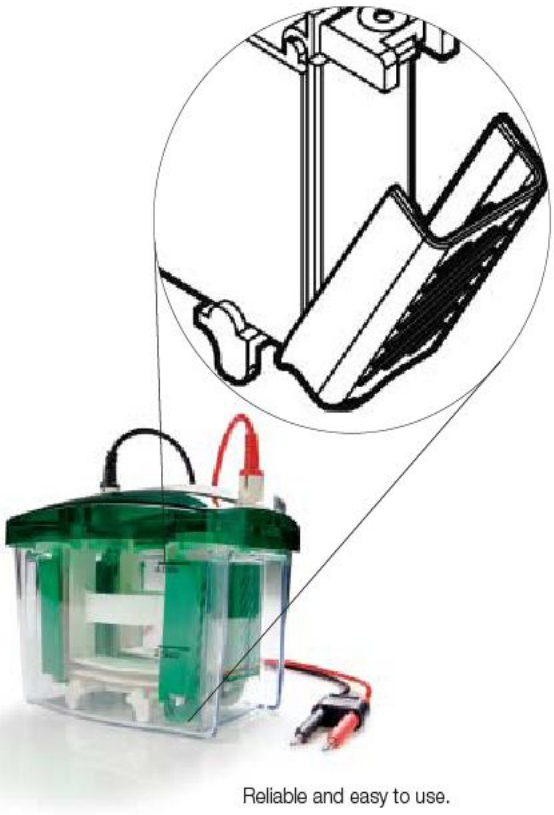
- Error-proof design to ensure correct polarity and orientation

* U.S. patent 6,436,262.



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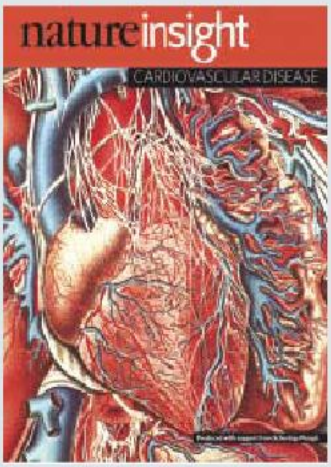
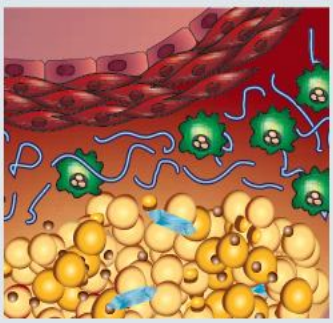


Cardiovascular disease is the most common cause of death worldwide and will become even more prevalent as the population ages. New therapeutic targets are being identified as a result of emerging insights into disease mechanisms, and new strategies are also being tested, possibly leading to new treatment options. Improving diagnosis is also crucial, because by detecting disease early, the focus could be shifted from treatment to prevention.



Study of how an atherosclerotic plaque forms, and the ability to visualize plaques *in situ*, is helping to improve diagnosis and treatment, pp. **904** and **953**.

CARDIOVASCULAR DISEASE

REVIEWS

PROGRESS

Replace cumbersome BrdU assays with one simple click

Accurate cell proliferation with Click-iT™ EdU

Measuring cell proliferation is a fundamental way to assess cell health, determine genotoxicity, and evaluate anti-cancer drugs. Bromodeoxyuridine (BrdU) cell labeling, followed by antibody detection, is the standard method to detect actively proliferating cells or cells in S-phase. Antibody detection of BrdU requires harsh treatments or nuclease digestion to facilitate epitope access. However, a new method from Invitrogen can detect proliferating cells with the nucleoside analog EdU (5-ethynyl-2'-deoxyuridine) without the need for antibodies or radioactivity.

Both the Invitrogen Click-iT™ EdU and BrdU assays follow similar workflows: cells or animals are given with the nucleoside analog, and the incorporated nucleoside is detected following fixation and permeabilization.

Where EdU and BrdU methods differ is in their simplicity of detection (Figure 1); detection of EdU is based on a click reaction—a copper-catalyzed covalent reaction between an azide and an alkyne. The dye azide's small size allows efficient detection of incorporated EdU under mild conditions. Standard aldehyde-based fixation and detergent permeabilization are sufficient for the Click-iT™ detection reagent to gain access to the DNA. This is in contrast to BrdU assays, which require DNA denaturation (using HCl, heat, or DNase) to expose the BrdU so that it may be detected with an anti-BrdU antibody.

By eliminating DNA denaturation, additional targets can be easily and reliably investigated. BrdU assay's DNA denaturation protocols using HCl and methanol can damage cell morphology and antigen recognition sites. And although milder than HCl, DNase denaturation can destroy your ability to perform cell cycle analysis. Finding the right balance between sufficiently denatured DNA and adequate amounts of dsDNA for the cell cycle dye to bind is difficult. With Click-iT™ EdU, however, content-rich results are now truly easy to obtain. You not only accurately measure proliferation of individual cells by flow cytometry, microscopy, or high-throughput imaging, but also simultaneously detect cell cycle, intracellular, and extracellular targets (Figure 2) in significantly less time than with the BrdU method.

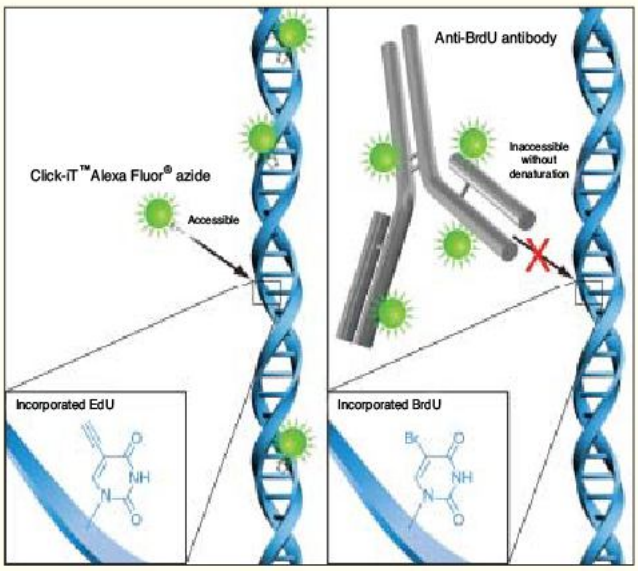


Figure 1—Detection of the incorporated EdU with the Alexa Fluor® azide versus incorporated BrdU with an anti-BrdU antibody. The small size of the Alexa Fluor® azide eliminates the need to denature the DNA in order for the detection reagent to gain access to the nucleoside.

Click chemistry makes biodiscovery a snap

To EdU-cate yourself further about this innovative new technology, visit www.invitrogen.com/edu.

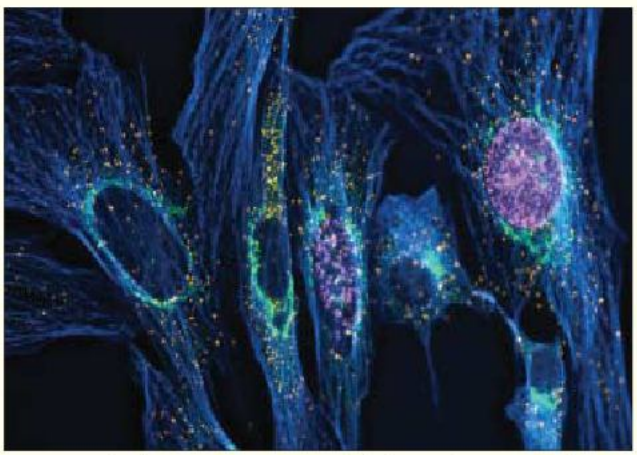


Figure 2—Multicolor imaging is a snap with Click-iT™ EdU. Muntjac cells were treated with 10 µM EdU for 45 minutes. EdU incorporated into newly synthesized DNA was detected with the far red–fluorescent Click-iT™ EdU Alexa Fluor® 647 Imaging Assay Kit (C10085). Tubulin was labeled with an anti-tubulin antibody (A11126) and visualized with an Alexa Fluor® 350 goat anti–mouse IgG antibody (A21049). The Golgi complex was stained with the green–fluorescent Alexa Fluor® 488 conjugate of lectin HPA from *Helix pomatia* (edible snail) (L11271), and peroxisomes were labeled with an anti-peroxisome antibody and visualized with an orange–fluorescent Alexa Fluor® 555 donkey anti–rabbit IgG antibody (A31572).

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ARTICLES

LETTERS

NATURE ONLINE

ADVANCE ONLINE PUBLICATION

PUBLISHED ON 13 FEBRUARY 2008

Gamete formation without meiosis in *Arabidopsis* M Ravi, M P A Marimuthu & I Siddiqi
doi:10.1038/nature06557

Facultative cheater mutants reveal the genetic complexity of cooperation in social amoebae L A Santorelli *et al.*
doi:10.1038/nature06558

PUBLISHED ON 17 FEBRUARY 2008

Proteomic analysis of active multiple sclerosis lesions reveals therapeutic targets M H Han *et al.*
doi:10.1038/nature06559

Regulation of progenitor cell proliferation and granulocyte function by microRNA-223 J B Johnnidis *et al.*
doi:10.1038/nature06607

Shotgun bisulphite sequencing of the *Arabidopsis* genome reveals DNA methylation patterning S J Cokus *et al.*
doi:10.1038/nature06745

PUBLISHED ON 20 FEBRUARY 2008

Hax1-mediated processing of HtrA2 by Parl allows survival of lymphocytes and neurons J-R Chao *et al.*
doi:10.1038/nature06604

Structure of the DNA deaminase domain of the HIV-1 restriction factor APOBEC3G K-M Chen *et al.*
doi:10.1038/nature06638

The inflammasome recognizes cytosolic microbial and host DNA and triggers an innate immune response D A Muruve *et al.*
doi:10.1038/nature06664

A peptide deformylase–ribosome complex reveals mechanism of nascent chain processing R Bingel-Erlenmeyer *et al.*
doi:10.1038/nature06683

Proline-catalysed Mannich reactions of acetaldehyde J W Yang, C Chandler, M Stadler, D Kampen & B List
doi:10.1038/nature06740

BRIEF COMMUNICATIONS ARISING

PUBLISHED ON 21 FEBRUARY 2008

Arising from 'Low Atlantic hurricane activity in the 1970s and 1980s compared to the past 270 years' by J Nyberg *et al. Nature* **447**, 698–701 (2007).
Is recent major hurricane activity normal? U Neu doi:10.1038/nature06576
Reply: J Nyberg *et al.* doi:10.1038/nature06577

Arising from 'Producing primate embryonic stem cells by somatic cell nuclear transfer' by J A Byrne *et al. Nature* **450**, 497–502 (2007).
Genotyping of *Rhesus* SCNT pluripotent stem cell lines (Corrigendum)
D S Cram, B Song & A O Trounson doi:10.1038/nature06759

GO DUTCH ON THE PODCAST

This week's cover image represents a crater on Mars — or rather a crater in a sand pit in Holland that's mimicking martian conditions. Hear all about it on the latest podcast, as well as rubber bands you can mend (check out the video showing it done), the genetic baggage that supports the 'out of Africa' hypothesis and the emerging-disease hotspots that we should be monitoring.
www.nature.com/podcast

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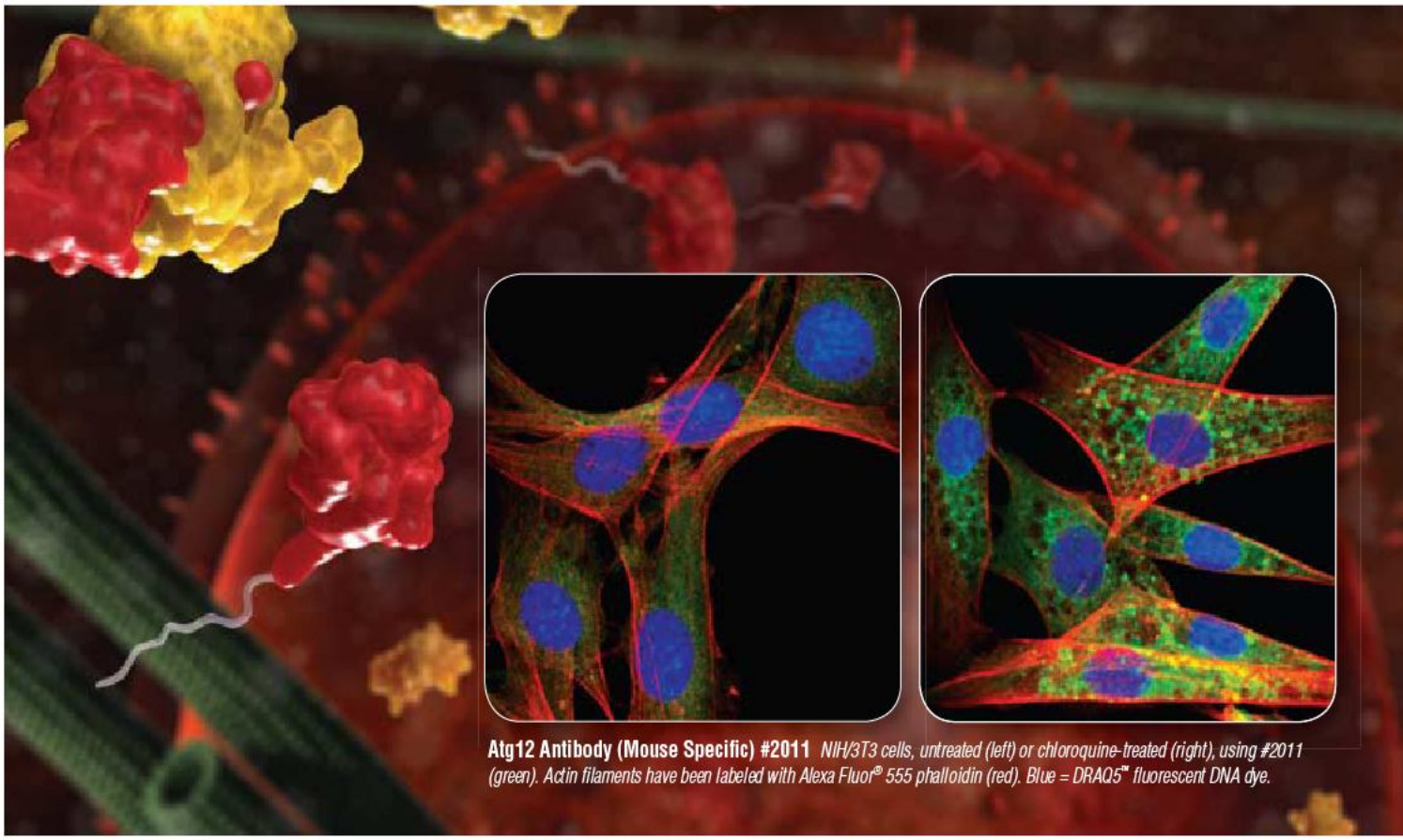

Atg12 Antibody (Mouse Specific) #2011 *NIH/3T3 cells, untreated (left) or chloroquine-treated (right), using #2011 (green). Actin filaments have been labeled with Alexa Fluor® 555 phalloidin (red). Blue = DRAQ5™ fluorescent DNA dye.*

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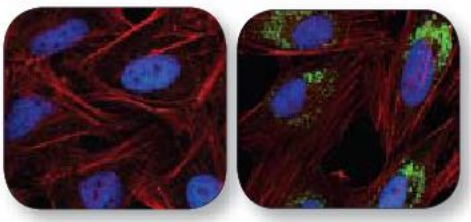

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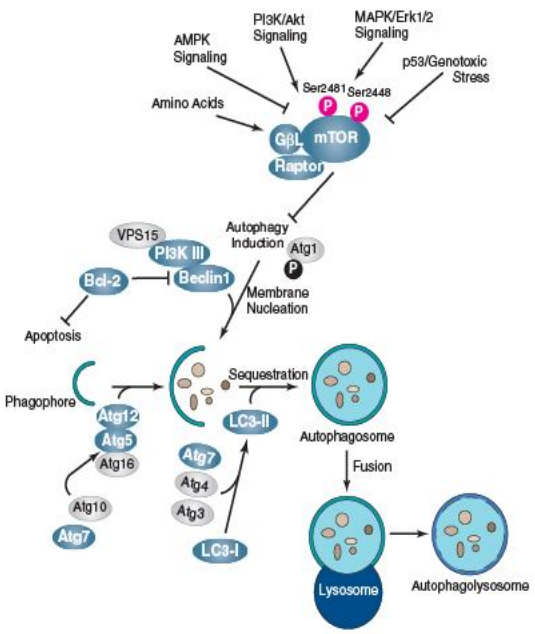
HeLa cells, untreated (left) or chloroquine-treated (right), using #2775 (green). Actin filaments have been labeled with Alexa Fluor® 555 phalloidin (red). Blue = DRAQ5™ fluorescent DNA dye.



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THIS ISSUE

GREENER BIOFUELS The honeymoon with biofuels is over. Environmentalists have been successful in spreading the message that fuels such as bioethanol are not so green after all, and rising food prices make the point dramatically. Can biotechnologists save the day with next-generations biofuels, such as ethanol produced by microbes? Jeff Tollefson asks the questions. [News Feature p. **880**]

LIFE CLASS In *The Living Cosmos*, Chris Impey outlines current thinking on how and where we should look for signs of extraterrestrial life. In its 50-year history the science of astrobiology — or exobiology — has made great strides, fuelled by a series of successful Solar System probes. But, as Bruce Jakosky's book review points out, the future of the field will depend on the development of new technologies. Though if a Mars sample return mission does find a slot in NASA's post-2010 plans, perhaps a new edition of *The Living Cosmos* will be on the cards. [Books & Arts p. **894**]

GETTING IT TOGETHER Though scientist can speak unto scientist in catch-all social networks like MySpace and Facebook, there is much to be said for smaller more specialized networks. Virginia Gewin talked to users and network organizers of some of the current batch of scientific and medical systems — including Chemical Forums, Doctors.net, NanoHub, Nature Network and PrometeoNetwork — to find out what they can provide that the more general networks cannot. [Naturejobs p. **1024**]

SUN SEEKERS The Solar Impulse aircraft, now under construction in Switzerland, is the prototype for a record-breaker. Pilots Bertrand Piccard and Andre Borschberg plan to be the first aeronauts to complete

SOLAR IMPULSE/EPFL CLAUDIO LEONARDI

Travelling light: a cockpit with a view.

a round-the-world trip in a heavier-than-air solar-powered aircraft. The 61-metre wingspan device is due for roll-out this autumn. Is there any practical point? Dump the pilots, and the answer could be yes. Pilotless aircraft are being developed for observation and telecommunications applications, and Solar Impulse hopes to prove the technology. [News Feature p. **884**]

Understanding how surface water flow could have produced the observed deltas and alluvial fans on the surface of Mars is fundamental to understanding the history of water on the planet. Flow duration in particular is an important factor, but to date, estimates for the longevity of martian hydrologic events have varied erratically, from decades to millions of years. Now, in a series of experiments here on Earth, in the Eurotank facility at Utrecht University, the characteristic morphology of martian stepped or terraced deltas has been recreated. The findings suggest that the stepped fans were formed by sudden release of water from subsurface storage, rather than by surface precipitation. In the conditions prevailing on Mars, this morphology is consistent with a single basin-filling event taking tens of years, and may have required an amount of water comparable to that discharged by a large terrestrial river about the size of the Mississippi. The cover image is a photo composite of a 4 mm-per-pixel digital terrain model of an experimentally formed crater from the Eurotank. [Letter p. **973**; www.nature.com/podcast]

Self-mending rubber

When a rubber-band breaks, that's it: time to get another one. But a remarkable new material described in this issue behaves rather differently. Consisting of molecules containing three different functional groups that form multiple hydrogen bonds, the molecules associate to form a 'supramolecular rubber' containing both chains and cross-links. The system shows rubber-like behaviour, that is, recoverable extensibility when stretched to several times its original length. In contrast to conventional rubbers made of macromolecules, these systems when broken or cut can self-heal when the fractured surfaces are brought together at room temperature. The new material can be synthesized from simple ingredients — fatty acids and urea — and once synthesized it is readily reprocessed. In its current form supramolecular rubber has slow strain recovery and it 'creeps' under stress, but by adjusting the starting ingredients, a spectrum of properties is attainable. [Letter p. **977**; News & Views p. **895**; www.nature.com/podcast]

Genetic baggage check

The analysis of genome-wide patterns of variation in human populations can provide genetic evidence of patterns of human migration and adaptation across the world. Two contrasting papers in this issue illustrate the power of the method. By combining a large number of datasets, Lohmueller *et al.* obtain precise estimates of the number of deleterious mutations carried by each of 15 African-Americans and 20 European-Americans, resequenced across 11,000 genes. They find that individuals with a European background have more potentially damaging mutations lurking in their genomes than those with an African background. This is interpreted as a genetic legacy from the 'out-of-Africa' bottleneck that accompanied the peopling of Europe. [Letter p. **994**; www.nature.com/podcast] Jakobsson *et al.* take a broader snapshot of human variation by examining 29 populations in the Human Genome Diversity Project. They obtain genotype data for over 500,000 markers in the human genome. Echoing the study of Americans with African and European backgrounds, these data reveal increasing linkage disequilibrium with increasing geographic distance from Africa. [Letter p. **998**]

An organelle with history

The apicomplexans are protozoan parasites of animals, including the pathogens causing malaria, toxoplasmosis and other human diseases. Most apicomplexans contain an unpigmented chloroplast remnant — the apicoplast — that is essential for the parasite's survival. It is thought that photosynthetic genes were lost during organelle evolution, but no descendant of a 'photosynthetic apicomplexan' with a photosynthetic plastid was known. Now an organism isolated from a stony coral in Sydney Harbour comes close to fitting that bill. The alga lives in association with corals, but can be cultivated as a free-living organism. It is phylogenetically related to Apicomplexa and its chloroplast features a genetic novelty — the presence of photosynthetic genes in combination with the use of the UGA codon to encode tryptophan — the latter being characteristic of apicoplasts. [Article p, **959**; News & Views p. **896**]

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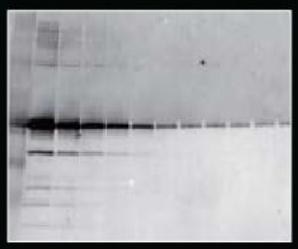

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Filter:BPFR700,
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Fluorescence detection of AlexaFluor® 750 Western blotting
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Filter	IP	LPB LPG IP	IP BPFR 700 BPFR 800
Photomultiplier	PMT1	PMT1	PMT1 PMT2
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Insulin resistance

The modification of nuclear and cytoplasmic proteins by *O*-linked *N*-acetylglucosamine (*O*-GlcNAc) is emerging as a key regulator for many cellular processes. One suspected role is as a nutrient sensor, linked to glucose flux through the hexosamine biosynthetic pathway. A study of the role of *O*-GlcNAc in the response to glucose flux reveals a new type of lipid binding site on the enzyme *O*-GlcNAc transferase (OGT): on insulin stimulation, the lipid phosphatidylinositol 3,4,5-trisphosphate binds to OGT, recruiting it to the plasma membrane. OGT then decorates insulin signalling pathway proteins with sugars, impeding their activity and dampening the insulin response. Overexpression of OGT in the liver of mice causes insulin resistance and dyslipidaemia. Abnormal *O*-GlcNAc modification of insulin signalling may therefore contribute to insulin resistance, obesity and type-2 diabetes. [Article p. **964**]

Hydrogen at speed

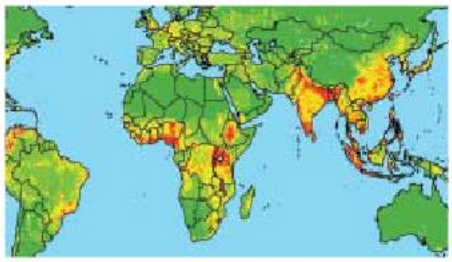
The extrasolar planet HD 209458b is surrounded by a vast cloud of atomic hydrogen, covering a region larger than the stellar disk. A spectral line observed when the planet passed in front of its host star revealed the presence of high-velocity atomic hydrogen a great distance away from the planet, and this was interpreted as hydrogen atoms escaping from the planet's exosphere and being accelerated by stellar radiation pressure. Starting from the fact that energetic neutral atoms are seen in the Solar System wherever energetic ions from the solar wind encounter a neutral planetary atmosphere, Holmström *et al.* have developed an alternative model. In this, stellar wind particles pick up electrons from neutral hydrogen in the planet's exosphere to generate energetic neutral atoms. [Letter p. **970**]

A new angle on flight

The quest to understand the origin of flight from the study of fossils and modern birds has spawned two distinct camps: those that think early birds took off from the ground, and those that think they started to fly by falling from trees or cliffs. The observation of birds in flight has not been very helpful, because wing movements vary so much depending on what the birds are doing. Now by filming chukars (a quail-like ground bird) in action, Dial *et al.* show that our confusion is a matter of perspective. If the line of the vertebral column is the reference frame, the wings appear to move according to particular behaviours. But if instead the angle that the wings make with the ground (gravity) is considered, it is found that this angle occupies a narrow range, irrespective of what the body is doing. Thus the flight stroke was a matter of learning to flap at a particular angle, irrespective of whether the protobird took off from the ground or jumped from a great height. [Letter p. **985**]

The next new disease

Emerging infectious diseases are a major threat to health: AIDS, SARS, drug-resistant bacteria and Ebola virus are among the more recent examples. By identifying emerging disease 'hotspots', the thinking goes, it should be possible to spot health risks at an early stage and prepare containment strategies. An analysis of over 300 examples of disease emerging between 1940 and 2004 suggests that these hotspots can be accurately mapped based on



Hotspots: mapping new vector-borne diseases.

socio-economic, environmental and ecological factors. The data show that the surveillance effort, and much current research spending, is concentrated in developed economies, yet the risk maps point to developing countries as the more likely source of new diseases. [Letter p. **990**; News & Views p. **990**; www.nature.com/podcast]

Neural stem cells in learning

The production of new neurons occurs in the adult brain, and appears to be influenced by external stimuli such as learning, exercise and stress. But it is not clear how the process is regulated or whether it is important for brain function. New work in knockout mice lacking the orphan nuclear receptor TLX, which is expressed in neural stem cells, suggests that adult neurogenesis plays a pivotal role in learning and memory. The mice had reduced stem-cell proliferation, and a marked decrease in spatial learning. But since other behaviours such as fear conditioning were unaffected, the new neurons appear to have a selective contribution to brain functions. [Letter p. **1004**]

Countering ischaemia

The transcriptional regulator PGC-1α mediates many of the effects of exercise on skeletal muscle, including mitochondrial biogenesis and fibre-type switching. Now this protein has been found to activate a natural defence pathway that protects ischaemic tissues. PGC-1α is produced in response to hypoxia and nutrient deprivation, and it in turn induces VEGF to promote blood vessel formation. This pathway is separate from the hypoxia response pathway involving hypoxia inducible factor and may provide a novel therapeutic target for treating ischaemic diseases of the heart, brain and limbs. [Letter p. **1008**]

Abstractions

LAST AUTHOR

A Holliday junction is a fleeting four-way crossover that occurs between DNA strands of the two chromosomes that make up a pair, allowing the reciprocal exchange of genetic information during a process known as homologous recombination. This mechanism was first proposed in 1964, and so far, only proteins from prokaryotes — organisms whose cells lack a nuclear membrane — have been shown to promote the formation of this type of DNA structure. On page 1018, Hiroshi Iwasaki of Yokohama City University in Japan and his colleagues demonstrate that similar proteins from eukaryotic — or nucleated — cells from yeast and humans promote Holliday-junction formation. Iwasaki spoke to *Nature* about why he finds the Holliday junction so intriguing.

You have worked on Holliday junctions for the past two decades. Why?

Holliday-junction formation is dangerous for cells, because their DNA must be cut in order to be rearranged. But this step is necessary for all organisms, not only to generate genetic diversity, but to repair damaged DNA — for example, in double-strand breaks. I find this very interesting. In 1991, we identified an enzyme that disassembles the Holliday-junction structure in the bacterium *Escherichia coli*. After that, I was motivated to find such an enzyme in eukaryotic cells, because no one had yet done so.

Did you use a novel approach to do this?

Yes. DNA is polar, and in prokaryotes DNA-strand exchange proceeds in only one of two possible directions. We looked for DNA-strand exchange from both polarities in eukaryotes, and found that it runs in the opposite direction to that in prokaryotes. Without looking in both directions, we would not have found the protein activity responsible for eukaryotic strand exchange.

Is there more to learn about basic biological mechanisms?

Yes. Basic biological mechanisms can easily get overlooked in science, but they underlie so many processes. For example, induced pluripotent stem cells — which can develop into any of the body's cell types — obtained from skin cells are a hot topic at the moment, but the fundamental mechanism underlying their transformation from differentiated cells to stem-like cells is simply the regulation of transcription.

Where will your work go from here?

We still don't know the precise mechanisms by which a Holliday junction is formed and later disassembled by the enzymes we have identified. My goal is to uncover the entire mechanism of homologous recombination. ■

MAKING THE PAPER

Erin Kraal

Shifting sands suggest origin of mysterious martian landforms.

Sandboxes are not just for kids. Giant ones — such as the Eurotank flume facility at Utrecht University in the Netherlands — can be used to study landscape and river evolution. And not just on Earth: Erin Kraal travelled to Utrecht to investigate landforms on Mars. But her big discovery came while she was taking time out from this work to show two high-school students how the tank can be used to study the formation of alluvial fans — the wedges of sediment left by rivers when they enter a basin. A simple demonstration in the 12×5-metre sandbox offered an unexpected explanation for how the red planet's 'stepped' deltas may have formed.

On completing her PhD, Kraal, now a research scientist at the Virginia Polytechnic Institute and State University in Blacksburg, was awarded an international fellowship from the National Science Foundation to spend her postdoc year at a foreign institution. She chose the home of the Eurotank. Her interest lay in the formation of alluvial fans, which provide a record of surface water flow. Understanding how these formations were produced on Mars is key to establishing not only where water flowed, but whether it was present for just decades or for millions of years.

Particularly intriguing were images of the martian surface that showed stepped, or terraced, fans. These are unlike any alluvial fan seen on Earth — here, such deposits have a single steep edge. Although Kraal wasn't focusing specifically on the origin of these stepped deltas, they were rarely far from her thoughts.

During her year at Utrecht, the editors of *Copernicus*, a European online science journal for young people (www.journal-for-young-scientists.net), approached Kraal to ask whether she might teach two high-school students about the Eurotank. She jumped at the chance. Clad in rubber boots, the trio carved extraterrestrial landscapes into the sand. They built a mock crater, fed a river to its rim and created an alluvial fan. The simple experiment was filmed, turned into a video and posted on *Copernicus*.

Afterwards, Kraal quickly drained the lake that had formed, because others were eager to use the facility. She was left with stepped deltas just like the ones in the images. "We were freaking out," says Kraal. "It had been a seat-of-the-pants experiment and we hadn't made any measurements!" She immediately shifted her attention to developing control experiments, and recreating the crater and the unusual deposits.

On page 973, Kraal and her colleagues offer a model for the creation of martian stepped deltas. They propose that water was released suddenly from subsurface storage, carved out short canyons, cascaded over the rim of a crater, filled it, then rapidly drained away. These discharges, which would have been comparable in size to large rivers such as the Mississippi, would have to have occurred over a period of decades, not millions of years.

"We don't see stepped deltas on Earth," says Kraal. "It is hard to imagine a situation that would have such a rapid release of water, but even if such a delta did form, it would not be preserved because there is so much rain here." The group is now exploring what might have caused the water's rapid release on Mars. One possibility is a volcanic intrusion, which might have melted ice, mobilizing the water very quickly. ■

FROM THE BLOGOSPHERE

In the space of a few days, *Nature*'s Editorial on double-blind peer review (*Nature* **451,** 605–606; 2008) had gathered almost 50 comments on the Peer-to-Peer blog at http://blogs.nature.com/peer-to-peer/2008/02/working_doubleblind.html#comments.

The Editorial concluded that double-blind peer review (in which both authors and reviewers are anonymous) is unlikely to be used at *Nature*, but asked readers for their views. In a torrent of comments, a theme emerged among self-defined junior researchers that the current single-blind system is biased against them in favour of established investigators.

But "Bob O'H" performed a model calculation that suggests that double-blind review merely shifts the bias so that "the very famous" actually do better, as do "the very obscure"; the scientists who lose out are the ones in the middle. Another view expressed is that in journals with high rejection rates, reviews are of lower quality.

Would double-blinding affect review quality? Or would it result in more scientists declining to review for journals? Your comments are welcome! ■

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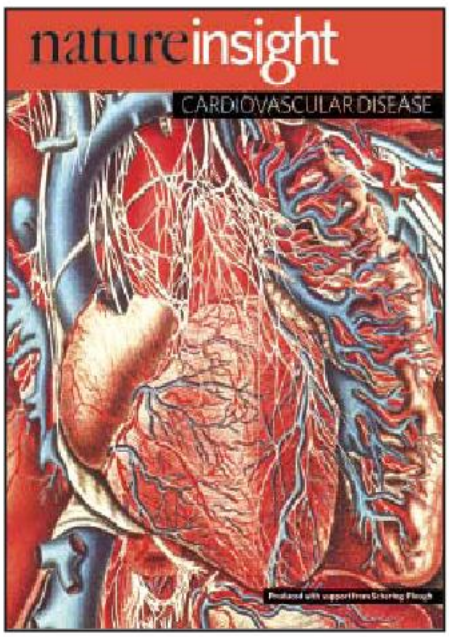
CARDIOVASCULAR DISEASE

Published in this issue of *Nature* and available FREE ONLINE for 6 months.



Cardiovascular disease is the leading cause of death worldwide. *Nature's* Insight on cardiovascular disease brings together up-to-date reviews on atherosclerosis, thrombosis, heart failure, cardiac arrhythmia, and congenital heart disease. These articles explore recent progress in understanding the underlying mechanisms of disease and discuss new strategies for treating patients, including the use of stem cells for heart disease. The articles also explore how new technologies for identifying disease biomarkers and for imaging may be able to detect early stages of heart disease, potentially changing the focus of therapy from treating disease to preventing it.

Read the Insight in this issue of *Nature* or access it free online for six months at:
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Technology Advancements Driving Biofuel Expansion

Given the growing concerns over global warming and a deteriorating environment, much attention is being paid to biofuels such as ethanol and biodiesel. Biofuels differ from fossil fuels in that they produce fewer exhaust gasses and less CO_2. They also make use of natural plant energy, which is environmentally friendly and abundantly available throughout the world. Research efforts are also ongoing to generate hydrogen, another alternate energy source, out of organic waste materials by help of bacteria.

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The Kyoto Protocol notes that biofuels used in motor vehicles are helping lower engine emissions. Any CO_2 exhaust produced by these fuels will be accounted for by the absorption of the CO_2 by the crops grown to produce more such fuels. Consequently, it can be said that the CO_2 gas from biofuels should not be considered as a greenhouse gas. In Japan, the Ministry of the Environment and other government ministeries are promoting the prevention of global warming by minimizing CO_2 generation.

Ethanol is produced through the fermentation of carbohydrates contained in various plants including the sugar in sugarcane, starch in corn and the cellulose found in rice. When ethanol is used to power motor vehicles, it is used as an additive in petrol, either in its pure form or as ETBE (ethyltertiary-butylether) a product of ethanol and isobuthene.

Many countries are now mixing ethanol with petrol, and at increasingly higher rates. In Brazil, for example, a government-regulated ratio of 20% to 25% ethanol to petrol per unit is the highest in the world for conventional petrol-based cars. There are also 100%-ethanol-powered cars available. In the USA an ethanol-to-petrol ratio of 10% is mandatory, though cars using a ratio of 10% to 85 % are also sold.

Conventional petrol-based cars are still in a major stream in the US and EU, while approximately half of the commercially new cars are dominated by flexible-fuel vehicles in Brazil, which make use of arbitrary percentages of ethanol in the petrol.

In Japan the maximum amount of ethanol added to petrol is restricted to 3%, and the production volume of ethanol is also less than in Brazil and the USA.

Brazil also produces and uses the world's highest volume of biofuels, in part because it is able to derive the world's highest energy efficiency from using these materials. A major reason for this high ratio is that it has replaced the burning of coal with corn pomace to drive some of the tubines producing the country's electricity. Brazil estimates that the energy efficiency of its overall use of biofuels is eight times that of petroleum. By comparison the estimated energy efficiency of biofuels used in the USA is 1.3 times that of petroleum.

Japan's Trial Use of Ethanol in Motor Vehicles Underway

The Petroleum Association of Japan has been conducting trial sales of bio-petrol or bio-ETBE, a mix of 3% ethanol and petroleum (C_4H_8, isobutene), since April 2007. Fifty petrol stations in the Tokyo metropolitan area are selling bio-ETBE, which is imported. Government regulations prevent the car industry from changing the percentage of this ethanol ratio.

The Petroleum Association plans to expand bio-ETBE usage to 16,000 kiloliters this year and the number of petrol stations will double to 100 sites. The Association will further increase this volume to 840,000 kiloliters in 2010 when volume sales are due to commence.

The law setting the maximum ratio of 3% bio-ethanol added to petrol was established in August 2003. According to a study conducted by the Ministry of Economy, Trade and Industry (METI) an alcohol concentration of 50% may corrode fuel systems in cars and cause accidents. (http://www.meti.go.jp/press/20070528001/shinnenryou.pdf)

During the North American International Auto Show held in Detroit this January, a Japanese automotive manufacturer exhibited a prototype car that runs on E100 or 100% ethanol, indicating that the technical challenges concerning its use in vehicles can be solved. The ethanol was supplied by British Petroleum in UK.

Non-Food Materials, a Must for the Future

Ethanol or ethyl alcohol is produced by fermentation of sugar obtained from certain kinds of plants. Plants can be categorized into C3 , C4 and CAM (crassulacean acid metabolism) plants based in part on the way they process CO_2. Most plants belong to the C3 category and these absorb CO_2 directly, while only C4 plants employ a CO_2 condensation mechanism that helps increase their photosynthesis capability. Corn and sugarcane are categorized as C4 plant and because they maximize their photosynthesis capability, their growth rate is rapid. More ethanol is produced from corn than any C3 plant.

Production of corn for fuel purposes, however, has been criticized for causing a shortage of land for growing food, and for an increase in food prices. It is possible to produce ethanol from wheat, rice and beet, as well as corn, but these materials are also used for food. One answer to this issue is to create a method that produces ethanol from the pomace of

sugarcane. The pomace of other plants such as rice and wheat are less efficient than corn, but these non-food materials can also become a source for producing ethanol in the future.

Biodiesel fuel is produced through transesterification of triacylglycerols (TAG) in virgin vegetable oil. It can also be produced from waste vegetable oils, but most commercial refiners are able to use these waste oils for other purposes.

USA: Ethanol Pioneer ICM Seeks to Produce Cellulosic Ethanol

Scott Kohl
Technical Director
ICM

"Ethanol is the best thing to happen to agriculture since the combine," says Dave Vander Griend, founder and president of ICM Inc., Colwich, Kansas, a design and engineering company that has helped pioneer the North American ethanol industry. He is referring to the surging demand for renewable fuels that has turned corn, the main ingredient in US fuel-grade ethanol, into a prized cash crop for farmers, rather than the government paying them cash not to grow corn in times of surplus.

Production figures speak for themselves. In 1980 the US produced a mere 175 million gallons (One gallon equals 3.785 liters) of ethanol, according to the Renewable Fuels Association. In 2001 the figure had jumped to 1.6 billion gallons annually and last year production hit 7.5 billion gallons, with another 5.7 billion gallons of capacity currently under construction.

"There are several major reasons for the dramatic increase," says Scott Kohl, technical director at ICM. "Farmers have seen that locally refining corn into ethanol adds value to their crop. So they have invested significantly to build local biorefineries." Surging oil prices are also helping make ethanol attractively priced. In addition, Kohl points out that adding ethanol to petrol helps reduce harmful car emissions, a plus for the environment and motivation for the federal and state governments to mandate its use in reformulated gasoline.

Along with engineering new plant construction, ICM also designs and manufactures the advanced equipment employed in them, including dryer systems, thermal oxidizers that eliminate over 99 % of airborne emissions, and wastewater treatment systems that allow plants to recycle their process water.

With demand soaring, ICM is stretching to keep up with plant and equipment orders. Of the 130 ethanol facilities currently in operation in the US, "Nearly 70 plants utilize ICM's patented process technology," says Kohl.

Instruments Are Key

When it comes to plant operations, ICM has turned to Shimadzu Scientific Instruments, Inc., Columbia, Maryland for assistance. "Smooth plant operations rely greatly on quality control equipment, explains Kohl. "Shimadzu supplies us with an HPLC (high performance liquid chromatography) system, which we use to monitor the critical fermentation process." He explains that the system meets ICM's needs because it is especially robust and reliable. "The software used to operate it is also user-friendly and easy for anyone to learn," Kohl adds.

A gas chromatograph from Shimadzu is another piece of equipment found in all ICM-engineered plants. This is used to monitor the final product to ensure it meets the specifications spelt out by customers of fuel-grade ethanol.

"It's not so much that Shimadzu's equipment is magic—other suppliers probably have similar products," says Kohl. "It is Shimadzu's customer service and support that is exceptional. You don't need a college degree to use their software, and if there is a problem with an instrument they respond immediately."

Rapid growth in the industry has raised challenges, however, like the current food vs. fuel debate. Critics say devoting cropland for ethanol feedstock production has reduced the land available for growing food, which in turn has led to rising food prices. While that is hotly contested, the search is on to find additional feedstock sources, with cellulose regarded as the most promising solution.

"Cellulose is found in virtually any plant matter," Kohl notes. "Trees, grass, corn stalks, even newspapers and much of our garbage have significant amounts of cellulose in them. Our goal is to innovate processes that can turn such non-food materials into fuel."

In 2005, a government interagency report found that US land resources are capable of producing a sustainable supply of 1.3 billon tons of cellulose materials a year, an amount equal to supplying 30% of the country's oil usage. (www1.eere.energy.gov/biomass/pdfs/final_billionton_vision_report2.pdf)

Now the race is on to turn this potential into a reality. Four years ago, ICM employed just four research scientists. Today it has almost 30. Besides developing technologies to make cheaper and better products for the industry in general, a majority of these researchers are now focused on the task of developing a practical process for converting cellulose into ethanol.

"To help us do this we are using the same types of Shimadzu instruments employed in our plant labs, as well as relying on some of their more advanced instrumentation," says Kohl. "An example of the latter is a GC/MS (gas chromatograph/mass spectrometer) that enables us to precisely identify many chemical compounds in liquids and gasses. Shimadzu is a great partner, even taking unusual research samples we've given them and analyzing them on more advanced instruments in their own labs."

Based on the progress ICM has made in its conversion goal, Kohl says he's now optimistic success is not far off. "We are currently building a small pilot plant to test out our process and already have plans to build a larger pilot plant within the next three years," he says. "Following that we could well be ready to move on to commercialization of cellulosic ethanol—probably within the next five to seven years."

Germany: Campa Biodiesel Ensures Quality of its Products

Udo Auerbach
Head of Quality Control Laboratory
Campa Biodiesel

German Campa Biodiesel located in Ochsenfurt close to Wuerzburg is producing 150,000 tons of biodiesel per year. Due to the fact that biodiesel is a natural product, its parameters and characteristics can change depending on the basic material used to produce it. To ensure the constant quality of biodiesel the DIN EN 14214 Standard was created, which defines the quality parameters of biodiesel.

Campa Biodiesel is a divison of Campa AG Holding in Ochsenfurt. The Holding company contains three divisions: Campa Süd in Straubing (Lower Bavaria) and Campa Biodiesel in Ochsenfurt produce biodiesel, while Campa Energie in Germany and Campa Iberia in Spain are distributing and selling it. Campa Biodiesel

started production in 2000, producing 70,000 tons of biodiesel a year and was one of the first producers of this type of fuel. The current capacity of the Ochsenfurt plant is 150,000 tons of biodiesel per year. In order to save the use of petrol-based diesel the government has mandated a content of 5 % biodiesel in diesel fuel. In 2009 it plans to increase this content to 7 % and possibly to 10 % further in the future.

"The manufacturing process of biodiesel is quite simple," says Udo Auerbach, head of Campa Biodiesel's Quality Control Laboratory. "Due to the fact that it is produced by transesterification, biodiesel comprises mono-alkyl esters of long-chain fatty acids derived from vegetable oils or animal fats." Consequently, it differs structurally from the alkanes and aromatic hydrocarbons found in petroleum-derived diesel. But because it is miscible with traditional diesel oils in any proportion, biodiesel is compatible with the existing diesel-fuel support infrastructure, which need no major modifications.

The choice of feed stocks for biodiesel manufacture depends on local availability and price. Biodiesel can also be produced from waste vegetable oils, however most commercial refiners currently find other uses for these oil wastes.

Biodiesel Complies With DIN EN 14214 Standard

To ensure the consistent quality of biodiesel in Europe the DIN EN 14214 Standard was created in 2003. It defines the quality parameters of the fuel and the ingredients used, as well as their specific quantities. Some of the quality parameters can change during production and so the standard requires a daily and a monthly analysis of the changeable parameters.

"To ensure the high quality of our biodiesel we decided to establish our own quality control laboratory and so looked for an appropriate gas chromatograph (GC)", says Auerbach. He contacted the headquarters of Shimadzu Europe Gmgh, Duisburg, Germany, because he knew that the Japanese scientific instruments supplier had worked closely with the standards body to simplify the measurement techniques used for determining the DIN EN14214 Standard. In 2004 Campa Biodiessel bought its first GC-2010 (Fig.1) from Shimadzu to analyse the quality of its biodiesel. The transesterification reaction of triacylglycerols (TAGs) in oils is most commonly done by reacting TAGs with methanol in the presence of a catalyst yielding the fatty acid methyl ester (FAME). During the process, monoacylglycerols (MAGs), diacylglycerols (DAGs) and other intermediate glycerols are formed. These, along with unreacted TAGs, may remain and contaminate the final product.

(Fig.1) The Shimadzu GC-2010 set-up in the Campa Biodiesel Ochsenfurt plant

The GC-2010 is specially designed for fast gas chromatography analysis and so increases productivity. It improves analytical efficiency by employing an advanced high pressure (970 kPa) flow control and high split-ratios as standard. Other features include extremely sensitive detectors, fast sampling times, and a high powered oven with high speed cooling capability. Thus it can double or even triple productivity compared to that of conventional instruments. It also incorporates excellent data reproduction capability (even for solvents with large vapor volumes such as acetone) by way of a newly developed injection system and highly sensitive detectors.

"Due to the success we had with the first Shimadzu GC-2010 we decided in 2006 to buy another one, which is mainly used to analyse our manufacturing process," says Auerbach."

Free glycerin, along with water, is a by-product of fatty acid methyl ester production. GC analysis of the glycerine concentration provides an effective measure of the fuel's quality. The American Society for Testing and Materials' (ASTM) D6584 method provides a test procedure for the quantitative determination of free and total glycerin in B-100 methyl esters using gas chromatography. Also, ASTM's EN14105 test method specifies a test procedure for the determination of free and total glycerol and mono-, di-, triglyceride contents by gas chromatography.

Of the 26 parameters to be analysed according to the DIN EN 14214 Standard, Campa Biodiesel uses the GC-2010 to analyse 10 of them. "We plan to buy other instruments from Shimadzu for analysing sulphur content, alkali metal, alkaline earth metal and phosphorous content," says Auerbach. "Analysing the sulphur content is necessary because it is found in some vegetable oils, while certain production processes use sulphuric acid." He also emphasizes the excellent customer service and support provided by Shimadzu. "Our close relationship with the Shimadzu Duisburg office and their prompt reaction to our needs has helped us realise a biodiesel of the highest quality."

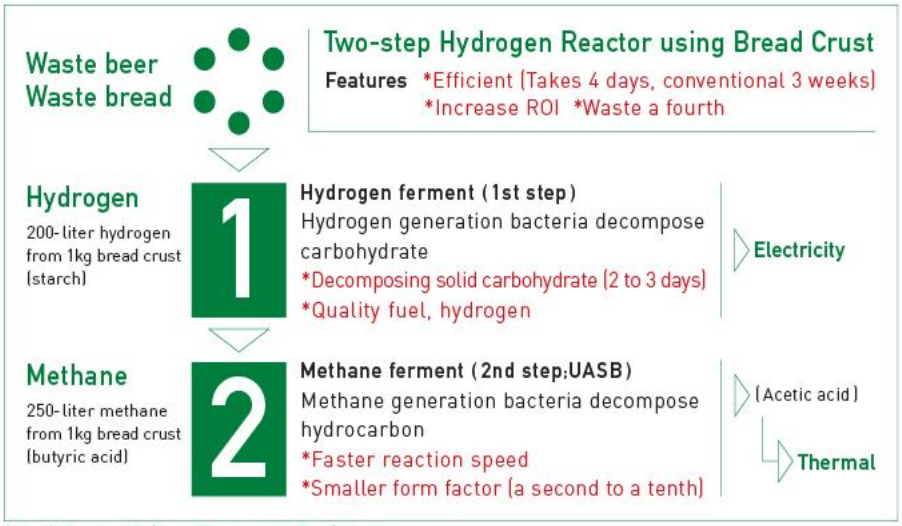
Generating Hydrogen with Bacteria

Tetsuo Hiraga
Manager
Corporate Strategy
Planning Dept.
Shimadzu

While ethanol and biodiesel fuels are used in car combustion engines, alternative emerging engine technologies are making use of electric power and hydrogen fuel cells. Recent research shows that hydrogen can be generated with the help of bacteria and this hydrogen in the form of fuel cells can be used to power a car engine directly without the need for fossil fuels (1). Tetsuo Hiraga, manager of Shimadzu Corporation's Corporate Strategy Planning Office, is one researcher making progress in developing technology to extract hydrogen from biomass.

Four years ago, Shimadzu, working jointly with Professor Naomichi Nishio of Hiroshima University, began development of a hydrogen generation system that uses bacteria to convert organic materials into hydrogen. The technology is a two-step production method that produces hydrogen and methane from organic waste materials remaining after food processing. Selected bacteria grown in a bioreactor consume the glucose contained in the food wastes and in the process generate hydrogen. The residue is then transferred to a second bioreactor where a different kind of bacteria consumes the remains, creating methane in the process (Fig.2).

The fermentation process that result in the creation of hydrogen is produced by bacteria, each measuring one micron in diameter. Professor Nishio first published a paper on the two-step generation of hydrogen and methane ten years ago (2). When Hiraga came across the paper, he proposed that Shimadzu work with Nishio to jointly develop the technology. The two-step fermentation process starts at the first bioreactor, where the bacteria consume the glucose contained in the fermented food waste and generate hydrogen as a by-product.



(Fig.2) Two-step Hydrogen Reactor using Bread Crust

(Fig.3) 900-liter reactor <top>
1-liter prototype reactor <bottom>

The residue of organic acids or acetic acid is transferred to a second bioreactor where it is consumed by a different kind of bacteria, which produce methane during the process (Fig.3).

Because Shimazdu is an analytic instrument manufacturer and not a fuel producer, Hiraga invited Sapporo Breweries, Ltd to help develop the technology, as the fermentation process is similar to the production of beer. He also sought the cooperation of the National Institute of Agrobiological Sciences and was able to obtain a grant from the Japanese government. The research team selected bread crust for the process material because it has high sugar content and can produce more hydrogen than other food wastes. Though some bread crust is used in pig feed, much is also discarded, making it an ideal source material.

Many kinds of bacteria coexist together. Consequently, bacteria other than the kinds that generate hydrogen and methane can also grow in the reactors. If certain types of bacteria enter a reactor, they may prevent the desired bacteria from increasing in sufficient numbers. Hiraga likens the various bacteria coexisting in the human body's intestines and the balance they maintain there, to the bacteria that can inhabit the two-step reactors. His goal is to find ways of maintaining the optimum number of the desired hydrogen-producing bacteria needed to make the process efficient.

One answer is to operate the reactors continuously, so as not to destroy the desired bacteria. The researchers took another step forward when they developed ways to enrich the cultures that produce the desired bacteria. As a result, the researchers have achieved some of the highest rates of hydrogen generation in the world: 2.5- to 3.5-mol hydrogen from 1-mol glucose. In practical terms the two-step technology produced 200 liters of hydrogen and 250 liter methane from 1kg of bread crust.

A Decision in Three Years

The two-step technology takes only four days to produce hydrogen and methane, compared to the 20 days it takes to produce methane alone by other means. Despite such success, Hiraga points out that though Shimadzu is an analytical instruments manufacturer producing a wide product portfolio, it has no experience in developing fuel processing technologies. So the company will decide in the next three years if the technology should be commercialized. Given that the first target application would likely be generating hydrogen for fuel cells, Hiraga says that commercial viability will depend on what the demand for fuel cell technologies is in that time frame.

The joint project with the National Institute of Agrobiological Sciences, Hiroshima University and Sapporo Breweries ended in April 2007. Now Hiraga is preparing to head a new three-year project with the help of a grant from the Ministry of the Environment. He will test the feasibility of using the two-step method for mass producing hydrogen in Hiroshima, and will set up a research facility adjacent to a bread factory there in order to facilitate easy access to the bread crust and other waste food materials the factory can supply.

Hiraga has chosen Hiroshima because Mazda, one of Japan's leading automotive manufacturers, is based there. Mazda has developed a prototype hybrid engine that employs both a petrol engine and a hydrogen engine housed in the RX-8 Mazda's proprietary rotary-engine-driven car. The hybrid car can switch from petrol to hydrogen power at the press of a button. However, Mazda has no plans to commercialize the hybrid vehicle anytime soon because the infrastructure to support hydrogen-powered vehicles, including fuel stations is non-existent. Nevertheless, Hiraga believes that as concern over the environment grows, cities will form eco-friendly and energy self-sustaining communities and these will facilitate the adoption of hydrogen-generated energy. He expects Hiroshima will become such a city.

(1) Rachman M.A.& Nishio,N. Enhanced Hydrogen Production in Altered Mixed Acid Fermentation of Glucose by Enterobacter Aerogenes. *Journal of Fermentation and Bioengineering* Vol,83,No.4,358-363 (1997).

(2) Nishio,N., Nakashimada,Y., Mitani,Y. & Hiraga,T. Hydrogen-Methane Two-Stage Fermentation (Hy-Met Process) for Anaerobic Waste Treatment. 15th World Hydrogen Energy Conference June 27 - July 2, 2004.

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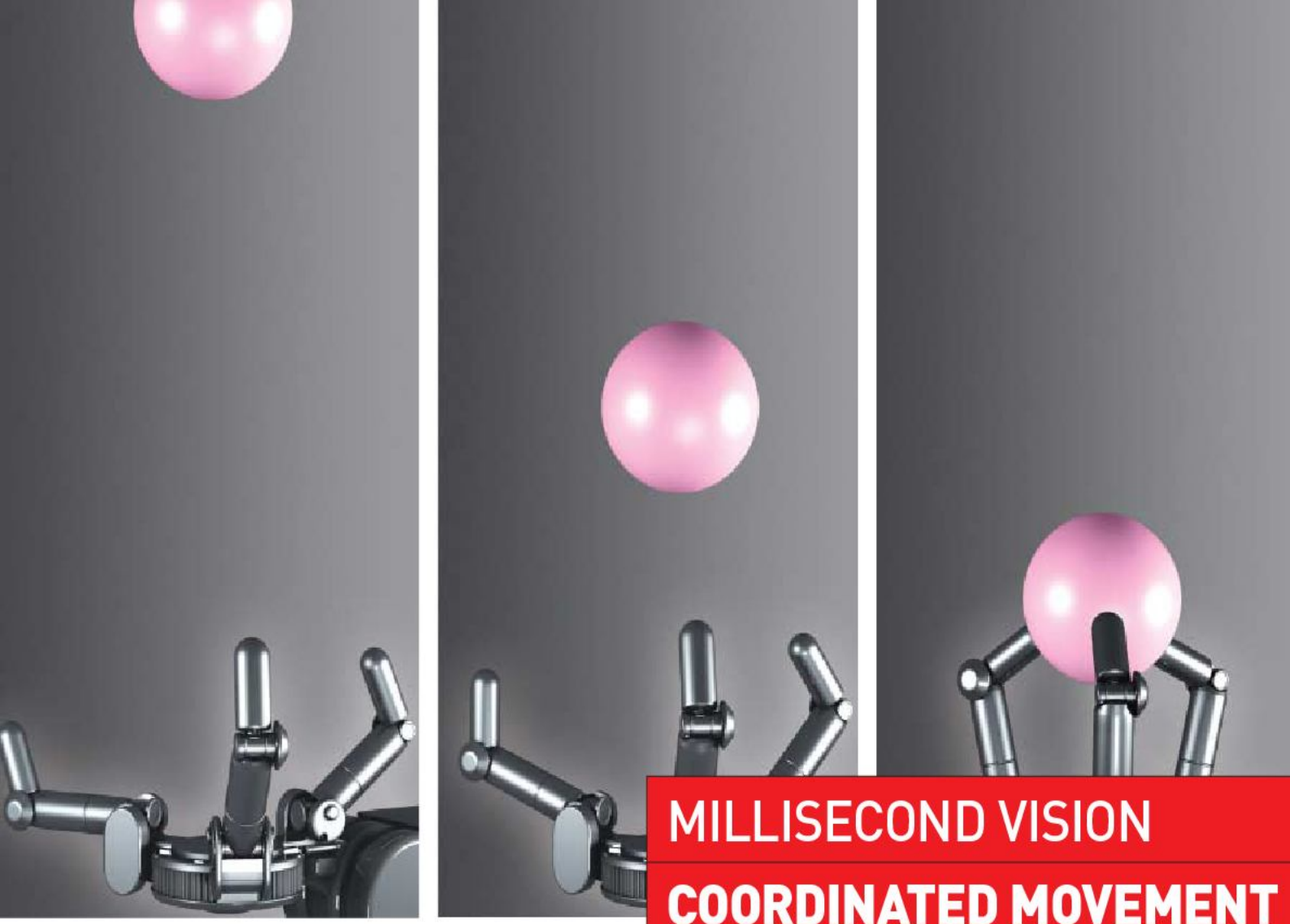

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One woman is still not enough

Japanese science needs its women more than ever. Why doesn't it treat them accordingly?

Seven years ago, Mitiko Go, a biophysicist then at Nagoya University, told *Nature* about a disturbing experience she had had at a meeting of the university's Division of Biological Science (see *Nature* **410**, 404–406; 2001). The academics were considering a female applicant for a vacant chair, and one male member said: "I'm sorry to have to say this in front of Dr Go, but one woman is enough."

Go thought she might be scolded for relating the story (and indeed she says she was accused of "tarnishing the honour of the university"), but she was about to retire from the university and felt the time had come to say something radical.

Times have changed. Far from retiring, Go is now president of the prestigious Ochanomizu University in Tokyo and a member of the Council for Science and Technology Policy, the country's highest science body, which is chaired by the prime minister. Go and others have implored the government to do more in support of women. The science and education ministry has responded.

Funding regulations, for example, now allow women on extended maternity leave to postpone grants rather than losing them. But the biggest change came in 2006 with a new budget to promote women in science, which has already jumped from ¥665 million (US$6.2 million) in 2006 to ¥1.8 billion this year. It supports programmes that encourage girls at high school to get into science. And it includes special competitive grants for 80 women returning to science after giving birth.

The biggest chunk of cash goes towards the development of 'model programmes'. Each year since 2006, the science ministry has funded ten new centres to the tune of ¥50 million per year to develop comprehensive programmes for the support of women scientists. Last week, women, and some men, from across the country met at Ochanomizu University to discuss the progress of these programmes. Princess Akishino attended and the mood was upbeat. Posters were sprinkled with phrases such as "Girls, be cheered". One logo features a woman jumping a hurdle. "The change has been epoch-making," says Go.

But there is something that remains token about all these gestures. Ochanomizu is the only one of Japan's 87 national universities with a female president. Only four have female vice-presidents. Go is one of two female members of the 15-member Council for Science and Technology Policy. Slight advances over the past few years have brought the ratio of women scientists to 12.4% of the total — a paltry number that pits Japan against South Korea at the bottom of developed nations and far below the European Union and US averages of about 30%.

It's not just the numbers. Visit most Japanese labs or go to academic conferences, and the women will generally recede into the background. Are their opinions being sought? Are their creative energies being tapped? There are no numbers on this, but the answer is undoubtedly 'no'.

"Making women active members of the scientific workforce means rethinking the work–life balance."

Japan needs its women like never before. There are fewer students than available university seats and a trend away from mathematics and science among students. The society is greying, and there remains an unwillingness to open the borders to foreigners on a large scale.

Many problems affect Japanese women and men alike. Original ideas from women are less likely to be taken seriously — but junior laboratory members in general are not encouraged to risk being wrong, to offer outlandish (aka creative) ideas. And no matter how much economic support there is for mothers, dirty looks, snide comments and a general attitude of unspoken disrespect aimed at one who leaves at 5 or 6 p.m. — even if it is to pick up the kids — will make lab life difficult. Making women active members of the scientific workforce means rethinking the work–life balance in general.

As part of her model programme, Go encourages all researchers at Ochanomizu to work 9 to 5. To do so, she has changed rules and faculty meeting schedules. This is by no means a revolution. But it may be a step in undoing a culture that has handicapped Japan by keeping roughly half its creativity under wraps. Too bad it can't happen faster.■

Forward with biofuels

Cellulosic biofuels are part of an emerging US energy policy, from which other regions can learn.

The energy law signed on 19 December by President Bush lays out a bold mandate for biofuels. As well as broadly ensuring that the United States remains home to the largest biofuels industry in the world in the coming decade, the law takes an important step forward by recognizing that all biofuels are not created equally. From 2016, refiners must begin to switch to cellulosic ethanol and other advanced biofuels that do not rely on corn sugars, and these fuels will have to meet new standards for reducing greenhouse-gas emissions compared with standard petrol.

That's the good news. The bad news is that, in the short term, this mandate will merely bolster the corn ethanol empire, which is far from ideal given the accumulation of evidence against the current generation of biofuels. The latest research suggests that any fuel that competes with food also encourages farmers around the world to expand their operations into native lands. Doing so causes a spike in emissions — from carbon once locked up in plants and soil — that might well outweigh the long-term benefits of the biofuels themselves.

Cellulosic ethanol, which can be produced from prairie grasses, municipal waste or just about any carbon-based materials, might well resolve these problems by expanding the feedstock away from food crops (see page 880). Exciting research is also under way on a third generation of synthetic biofuels — designer fuels secreted by

specially engineered microorganisms. But although such 'novel molecules' could resolve a host of infrastructure issues that are unique to ethanol, they don't amount to much more than fancy corn fuels unless the feedstock question is resolved. From a research and development perspective, the priority must remain cellulosic conversion.

Money and talent are flowing into the energy arena from both private and public sectors, but these technologies have yet to advance beyond the realm of pilot plants and press releases. The next few years will be critical as the industry tries to bridge the gap from demonstration projects to commercial-scale production. If the new biofuels fulfil their promise, they may well naturally supplant corn ethanol, in which case the mandate will have done what it is supposed to do. If not, Congress may need to step in again.

All of this should serve to remind policy-makers that the science of biofuels is still evolving, as are the tools for tracking greenhouse-gas emissions. It also underscores the need for sustained attention to energy issues (including energy efficiency, a doubling of which would enormously amplify the benefits of biofuels). That sounds like a truism but in fact would represent a novel and important shift from the episodic, crisis-driven attention paid to this issue in decades past. Fortunately this is starting to happen. When the United States enacted a comprehensive energy law in 2005, lawmakers touted it as the first major reform in 13 years. Congress passed another major bill in December, and global-warming legislation may well be just around the corner.

Across the Atlantic, European nations are struggling with the same issues — and still have time to learn from the United States' mistakes. Rather than promoting all biofuels, they should ensure that their policies support those second-generation technologies that will provide the biggest pay-off. The European Commission recently released a plan targeting biofuels for 10% of transportation fuels by 2020. Commission president José Manuel Barroso said the proposal would create "the most comprehensive and sustainable system anywhere" for certifying greenhouse-gas emissions from biofuels. The European Union and its member states need to ensure that they follow through. ■

Science in retreat

Canada has been scientifically healthy. Not so its government.

Comparisons of nations' scientific outputs over the years have shown that Canada's researchers have plenty to be proud of, consistently maintaining their country's position among the world's top ten (see, for example, *Nature* **430**, 311–316; 2004). Alas, their government's track record is dismal by comparison.

When the Canadian government announced earlier this year that it was closing the office of the national science adviser, few in the country's science community were surprised. Science has long faced an uphill battle for recognition in Canada, but the slope became steeper when the Conservative government was elected in 2006.

The decision in 2004 by the then prime minister Paul Martin to appoint a scientist for independent, non-partisan advice on science and technology was a good one — in principle. Arthur Carty, the chemist who secured the position, duly relinquished his post as president of the National Research Council Canada, which he had revitalized.

But his new office was destined to fail. The budget was abysmal and the mandate was vague at best. After winning power from the Liberals, the Conservatives moved Carty's office away from the prime minister's offices to Industry Canada. In 2007, the government formed the 18-member Science, Technology and Innovation Council (STIC). Told that the government would no longer need a science adviser, Carty offered his resignation. From March, the STIC will provide policy advice and report on Canada's science and technology performance. It can be expected to be markedly less independent: although it is stocked with first-class scientists and entrepreneurs, several government administrators also hold seats.

Concerns can only be enhanced by the government's manifest disregard for science. Since prime minister Stephen Harper came to power, his government has been sceptical of the science on climate change and has backed away from Canada's Kyoto commitment. In January, it muzzled Environment Canada's scientists, ordering them to route all media enquires through Ottawa to control the agency's media message. Last week, the prime minister and members of the cabinet failed to attend a ceremony to honour the Canadian scientists who contributed to the international climate-change report that won a share of the 2007 Nobel Peace Prize.

Harper sees himself as the leader of a 'global energy powerhouse' and is committing Canada to a fossil-fuel economy. More than 40 companies have a stake in mining and upgrading the bitumen from the oil sands in Alberta and churning out 1.2 million barrels a day. This activity generates three times as much greenhouse gas as conventional oil drilling. Emissions from Canada's oil and gas industry have risen by 42% since 1990.

There are deeper and more chronic problems for Canadian science. On the surface, funding for university-based research seems strong. The annual budgets for the Canadian Institutes of Health Research (CIHR) and the National Sciences and Engineering Research Council tripled and doubled, respectively, between 2000 and 2005. The government has also supported new science projects through government-created corporations such as Genome Canada and the Canada Foundation for Innovation, and has recruited and retained promising young scientists through the Canada Research Chairs programme.

But Genome Canada funds only half of the cost of a research project — scientists must seek the remaining cash from elsewhere. Last year, the CIHR was able to fund only 16% of the applications it received, and cut the budgets of successful applicants by a quarter, on average. And earlier this month, the country's top scientists and university officials warned that they were short of funds to operate multimillion-dollar big-science projects such as the Canadian Light Source synchrotron.

What's to be done? Canada has made good investments in its science infrastructure and its future research leaders. The present government might be dissolved after a vote of confidence next month, which could in itself lead to a change for the better. But in any circumstances, Canada's leading scientists can be public advocates, pointing to the examples of other countries in urging the government of the day to boost their country into a position of leadership rather than reluctant follower. ■

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RESEARCH HIGHLIGHTS

Lifting a whale

Phys. Rev. Lett. 100, 054502 (2008)
Engineers at Harvard University have worked out how the bumpy edge of humpback whales' pectoral fins helps the animals to perform underwater rolls and loops without stalling.

Stalling happens when a hydrofoil — in this case, a fin — climbs through a fluid at too steep an angle. This causes the flow to separate from the hydrofoil's upper surface, removing the low pressure there that causes lift.

Ernst van Nierop and his colleagues' calculations show that a bumpy fin inhibits separation of the turbulent fluid layer close to the fin surface, delaying stall to higher angles and making its onset more gradual than if the fin were smooth in shape. The lessons learned from humpbacks might be applied to wings, boats and turbine blades, they add.

P. ATKINSON/NHPA

CHEMICAL BIOLOGY

Adding an adaptor

Nature Chem. Biol. doi:10.1038/nchembio.73 (2008)
A team at the Medical Research Council in Cambridge, UK, has found a way to get cells to genetically encode the amino acid lysine with an acetyl group attached. The addition or removal of this chemical group influences a host of cellular processes, and this work paves the way for researchers to probe its involvement further.

In nature, there are 64 'codon' combinations encoding just 20 amino acids, so the code carries redundancies that synthetic biologists can exploit. Several teams have already created synthetic amino acids with no real biological significance.

Jason Chin and his colleagues used the same method of re-engineering a transfer RNA molecule, which acts as an adaptor linking each amino acid to its codon, to extend the code. But because acetylated lysines have a role in modifying how genes are expressed, and are found in p53, a protein that is associated with many cancers, this research should prove more useful.

The authors also found a way to prevent cells from removing the acetyl group after proteins had been synthesized.

ASTRONOMY

Distant inflow

Astrophys. J. 674, 151–156 (2008)
Large inflows of gas helped to fuel star formation in galaxies in the early Universe, according to a new model. Such galaxies thrived when the Universe was only 2 billion to 3 billion years old, and were hotbeds of star birth. Although astronomers have observed gas blasting out of them, measuring the inflow of gas has proved difficult.

To maintain high rates of star formation, gas must enter galaxies at roughly the same rate that it exits or forms stars, points out Dawn Erb of the Harvard-Smithsonian Center for Astrophysics in Cambridge, Massachusetts. Her model takes the Kennicutt–Schmidt law — widely used in studies of nearby galaxies to relate the density of gas in a galaxy to the amount of star formation — and considers its implications for distant galaxies.

The gas influx that the model predicts cannot be detected with current instruments. It may be the limiting factor in star formation, and thus important in the evolution of far-off galaxies, Erb adds.

GENOMICS

Inactive binding

PLoS Biol. 6, e27 (2008)
Researchers may have been misinterpreting the results of a method that is used to identify DNA regions involved in turning genes 'on' and 'off'.

The technique, known as ChIP-chip, isolates DNA sequences bound by proteins called transcription factors, which control gene expression. Michael Eisen and Mark Biggin at the Lawrence Berkeley National Laboratory in Berkeley, California, and their colleagues have demonstrated that transcription factors often bind DNA without changing gene expression.

They used ChIP-chip to study the binding sites of six proteins that control early embryonic development in the fruitfly *Drosophila melanogaster*. The proteins attached themselves to thousands of sites in the genome — a number that greatly exceeds that of the genes thought to be under these proteins' control. Further analysis indicated that the proteins bound less strongly to sites where they did not seem to affect the expression of neighbouring genes.

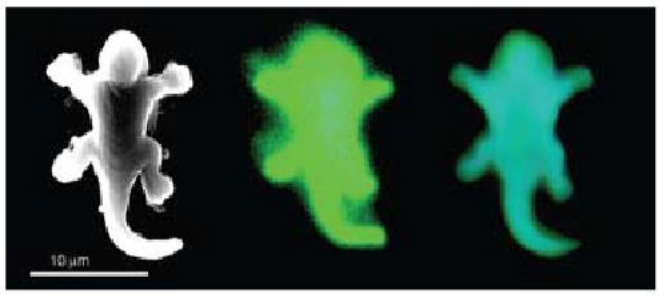
LITHOGRAPHY

Luminous lizards

Adv. Mater. doi:10.1002/adma.200702035 (2008)
Xuan-Ming Duan at the Chinese Academy of Sciences in Beijing and his colleagues have made fluorescent bull and lizard sculptures (pictured below) not much bigger than red blood cells. The tiny creatures luminesce either green or blue.

The team had already honed a technique to fashion three-dimensional sculptures, such as these animals, using a laser to pierce a translucent viscous resin. Where the laser is focused, it sets off a polymerization reaction that hardens the resin.

This time, the researchers mixed



WILEY-VCH

fluorescent nanoparticles into the goo. They found they could vary the sculptures' colours by altering the size of the nanoparticles and the tightness of the resin molecules' weave. Their animal creations are offered as proof of principle for a means of making miniature light-emitting electronics.

NANOTECHNOLOGY

Wires of code

Nature Nanotech. doi:10.1038/nnano.2008.4 (2008)
DNA's electrical properties are similar to those of graphite, report researchers in the United States who wired strands of DNA into electrical circuits and measured their conductivity. This is because both structures contain stacks of aromatic, or ring-shaped, organic molecules that have clouds of delocalized, floating electrons.

Colin Nuckolls at Columbia University in New York, Jacqueline Barton at the California Institute of Technology in Pasadena and their colleagues took a tiny electrical circuit made from carbon nanotubes and snipped it open. They then bridged the gap by attaching a stretch of DNA, modified with amine groups, through covalent bonds. They measured current flowing through the altered circuit, and compared it with the current that had flowed through the original nanotube circuit.

The findings help demonstrate that DNA could be used to turn biochemical processes into electrical signals on a very small scale.

OPTICS

Tiny holograms

Nature Photon. doi:10.1038/nphoton.2007.300 (2008)
Two scientists at Johns Hopkins University in Rockville, Maryland, have developed a way to create high-resolution three-dimensional holographic images of fluorescing samples under a microscope.

Typically, holograms are generated with a device called a laser interferometer, in which light from a reference beam interferes with that reflected by an illuminated object. But this technique is slow and can create only fuzzy holograms of microscopy samples.

Joseph Rosen and Gary Brooker used a technique known as Fresnel incoherent correlation holography, which can create a holographic interference pattern quickly, using light from a simple electric bulb. The duo captured the interference pattern with a digital camera and used computer software to recreate the hologram. Brooker says that the technique is simple, relatively inexpensive, and might one day be used to show the motion of microscopic samples in real time.

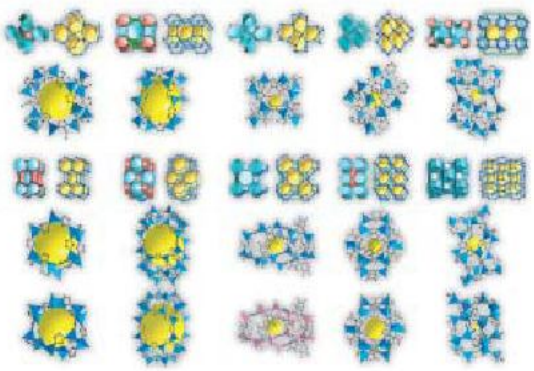
CHEMISTRY

Carbon suckers

Science doi:10.1126/science.1152516 (2008)
Using techniques pioneered in drug development, researchers have synthesized metal–organic 'sponges' with an "extraordinary capacity" for storing CO_2.

Rahul Banerjee and Omar Yaghi at the University of California, Los Angeles, and their co-workers created 25 crystals called zeolitic imidazolate frameworks (ZIFs) by bringing together tiny amounts of ingredients in thousands of separate microreactions on glass plates. The method, they say, allowed the creation of structural arrangements never before seen in ZIFs (pictured below). Of the frameworks they created, 16 had new compositions and 5 had new topologies.

Three of the sponges show an amazing affinity for separating CO_2 from CO, outperforming the two previously known ZIFs and the forms of activated carbon that are currently used, for example in detergents and for refining petrol.



O. Y. YAGHI

ENVIRONMENTAL SCIENCE

Leaden appetite

J. Wildl. Manage. **72**, 240–245 (2008)
During the elk-hunting season, common ravens (*Corvus corax*) in the Jackson Hole valley in Wyoming can have more than five times the concentration of lead in their blood than they do at other times of year.

Researchers Derek Craighead and Bryan Bedrosian of Craighead Beringia South, a non-profit conservation research outfit in Kelly, Wyoming, followed the birds for a 15-month period that included two hunting seasons. They think that hunters' lead bullets fragment into hundreds of tiny pieces when they hit animals such as elk, whose carcasses ravens later eat.

The authors argue that humans and other animals that consume hunted meat might also be ingesting unsafe levels of lead.

JOURNAL CLUB

Genevieve Almouzni
The Curie Institute, Paris, France.

An expert in chromosome organization considers yeast in a new light.

As somebody who studies how DNA is packaged so that it fits inside the nucleus, and how this protein parcelling adds to the information held in the sequence of DNA bases, my work has focused on frogs and mammals. Brewer's yeast (*Saccharomyces cerevisiae*) is one of the simplest organisms with nuclei. It has proved useful to researchers like me when considering subtle influences on gene expression that are also found in higher organisms.

But we have not found a yeast 'counterpart' for some mechanisms, such as those that rely on RNA to regulate gene expression. One example is RNA interference, by which genes are 'silenced' through destruction of the messenger RNA molecules that would otherwise convey protein 'recipes' from the nucleus to the cytoplasm. But this does not rule out similar effects on gene expression by other means, as Françoise Stutz and her colleagues at the University of Geneva in Switzerland have found (J. Camblong *et al. Cell* **131**, 706–717; 2007).

This team stumbled across silencing of a different sort when they left plates of yeast to divide for varying amounts of time. They found that older yeast cells expressed a gene called *PHO84* less than did younger cells, and that as the amount of mRNA encoding the Pho84 protein decreased, the level of an antisense (or mirror-image) version of this mRNA increased. A series of experiments led them to propose a mechanistic model in which tuning the RNA degradation machinery stabilizes the antisense transcripts, promoting modifications of chromatin — the DNA–protein complexes that make up chromosomes — and, in turn, regulating gene expression.

No one yet knows how common this effect is in yeast, nor whether it occurs in more complex life-forms. But this paper does serve as a lesson to revisit our assumptions.

Discuss this paper at **http://blogs.nature.com/nature/journalclub**

NEWS

Experts suspicious of 'splatellite' plan

A plan by the US government to shoot down an out-of-control spy satellite has been described as a cynical tit-for-tat move in response to China doing the same last year. Scientists and arms-control experts fear that the operation will create damaging debris and weaken international efforts to ban space weaponry.

On 14 February, officials from the Pentagon, White House and NASA announced plans to use a ship-based missile to strike the satellite as it passes roughly 240 kilometres overhead. The satellite, which belongs to the National Reconnaissance Office in Virginia, dropped out of control after its launch in December 2006, and would re-enter Earth's atmosphere around early March if no action were taken.

US NAVY PHOTOS

The US navy plans to shoot down an errant satellite with an SM-3 missile.

The strike is necessary to prevent the dispersal of around 450 kilograms of hazardous hydrazine thruster fuel onboard, according to James Jeffrey, assistant to the president and deputy national security adviser. If the fuel survived re-entry, it could be dispersed over an area of roughly 20,000 square metres, although "the likelihood of the satellite falling in a populated area is small," he says. "Nevertheless, if the satellite did fall in a populated area, there was the possibility of death or injury to human beings." The Pentagon denies that the shoot-down is to protect classified technologies on the satellite.

But scientists familiar with both satellite re-entry and the US missile defence system question the decision. The chances that the tank, which is 1 metre in diameter, will survive and strike land are extremely small, says Geoffrey Forden, a physicist at the Massachusetts Institute of Technology in Cambridge. "Most likely it will land in the ocean," he says. The reasons given for the plan "don't sound too credible to me", he adds. "I think they're doing it mainly to tell the Chinese that we can blow up a satellite too," says Jonathan McDowell, an astronomer at the Harvard–Smithsonian Center for Astrophysics in Cambridge, Massachusetts. "This gives the US cover to carry out a test."

The firing will probably take place in the coming weeks, although not before the return of the space shuttle Atlantis, which is expected back from the International Space Station on 20 February. David Wright, a physicist at the Union of Concerned Scientists, a non-profit organization in Cambridge, Massachusetts, says that he believes the station could be vulnerable to debris. But NASA administrator Michael Griffin says he is "very comfortable" with the decision.

Hitting the satellite could have serious consequences. In January last year, when China used an interceptor to destroy one of its own, obsolete weather satellites, the test littered more than 100,000 debris fragments throughout low-Earth orbit. Much of this hazardous debris will remain there for decades, posing a risk to other satellites.

The errant US satellite is at a much lower orbit than the Chinese one was, and therefore debris would be shorter lived and less likely to cross the path of other spacecraft. But it is also at least 2.5 times larger than the Chinese one, so it will create more debris. Furthermore, the cloud could behave unpredictably, says Wright.

The government plans to destroy the satellite using a ship-launched Standard Missile 3, or SM-3. The missile is designed to use a kinetic kill vehicle to ram incoming ballistic missiles, destroying them before they damage US targets. It is a smaller and slower device than the ground-based interceptors located at Fort Greeley in Alaska and Vandenberg Air Force Base in California. But it is better at intercepting targets, according to Forden. "They have had quite a bit of success with the SM-3," he says.

Travelling at 3–4 kilometres per second, the device would smash into the 2,250-kilogram satellite, which itself will be moving at roughly 8 kilometres per second. "At these speeds it is like setting off a huge amount of high explosive at the satellite," Wright says. Even without carrying explosives, the energy of the collision could boost fragments of the satellite into a higher orbit, creating hazards for other craft. "It sounds like a bad idea to me," he says.

The announcement came just two days after Russia proposed a treaty, backed by China, to ban the use of space weapons — including those used to destroy a country's own satellite — at an international conference on disarmament in Geneva, Switzerland.

The proposed US shoot-down would have far-reaching diplomatic implications. "If you do this," says McDowell, "you have converted your missile-defence system into a missile-defence and anti-satellite system."

"It would reinforce people's sense of the United States as being irresponsible," says Rebecca Johnson, executive director of the Acronym Institute for Disarmament Diplomacy in London. The United States has blocked a ban on space weapons for more than a decade on the grounds that it would interfere with its right to develop a missile-defence programme. Using that system to destroy an orbiting satellite would probably anger countries such as Russia.

McDowell says he thinks the shoot-down, following in the wake of China's test last year, will dramatically weaken already floundering efforts for a ban on space weapons. That in turn could be hazardous for satellites everywhere. "Just because the Chinese were idiots, doesn't mean that we have to be bigger idiots," he says. ■

Geoff Brumfiel, with additional reporting by Rachel Courtland

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Check your GPS at the border

Foreign researchers working in China are falling foul of laws restricting environmental monitoring and use of Global Positioning System (GPS) equipment. Geologists, botanists, environmental scientists and meteorologists have been affected. Even those who believed that they were within the law and were collaborating with Chinese researchers have lost data, been detained and had equipment confiscated.

According to the State Bureau of Surveying and Mapping, in 2006 there were 759 cases of illegal geographical surveys and mapping, many of which involved foreigners. And there have been 20 cases of illegal meteorological surveys by foreigners since 2000. Last year, two new stricter laws governing data collection were introduced to "protect national security": these were Measures for the Administration of Foreign-related Meteorological Sounding and Information, introduced in January; and Measures Governing the Surveying and Mapping in China by Foreign Organizations or Individuals, in March. The new laws require projects to be approved by the China Meteorological Administration or the State Bureau of Surveying and Mapping, in addition to the current approval processes, and stipulate that any data are gathered and interpreted jointly with Chinese counterparts.

Some experts believe that the stricter meteorological laws were introduced in reaction to a presentation of environmental data at a conference in Hong Kong in May 2006 that, according to the official Chinese press, "amplified China's environmental problems to draw more attention to their research, heedless to the fact that data collected in polluted areas doesn't represent the norm in China". The research had been carried out in collaboration with Chinese scientists, but Chinese officials say that the research was published "unilaterally", in violation of a previously agreed arrangement.

But the laws are not well known in China or abroad. Jefferson Fox of the East-West Center at the University of Hawaii, and his collaborator Jianchu Xu, of the Kunming Institute of Botany in Yunnan province, were stunned when in February 2007, local security officers dismantled their field stations in southwestern Yunnan province. The scientists were comparing field data with Landsat satellite data, in a project to assess the impact of rubber-tree monocultures on hydrology and land cover.

"Researchers have been detained and had equipment confiscated."

Some equipment was returned a week later, but many of the cables had been cut and underground soil-moisture sensors were lost, resulting in damage worth more than US$20,000 and curtailing the study halfway through. "We were just starting to get very good data," says Fox. "It was just a bad experience."

Ramón Arrowsmith, a geologist at Arizona State University in Tempe, was similarly disturbed when local officials came to his team's hotel by the Altyn Tagh fault, near the border between the Tibet and Xinjiang autonomous regions in western China in March 2007. With Chinese counterparts, Arrowsmith was studying the record of deformity — ridges and humps — along the fault line to understand the history of the Indo–Asian plate collision. The officials objected to his use of GPS equipment, the kind, Arrowsmith says, that can be purchased cheaply at a camping-equipment store.

Arrowsmith tried unsuccessfully to convince the officials that he was only measuring specific geological features relative to each other, not absolute topography. "It was scientifically very interesting, but from a general perspective, this was a really boring project," he says. Despite having received permission to do research through his visa application and from the local nature reserve, Arrowsmith and his team were detained in their hotel for 10 days before being allowed to leave China. "They were not abusive, but it was stressful," he says. "We're not used to being questioned."

The Chinese official media has reported copious other transgressors over the past year, including several Japanese groups. The meteorological administration says that there have been more than 40 such cases involving foreigners. Some of them lost equipment or faced fines ranging up to 80,000 yuan (US$11,000). The UK Royal Yachting Association, preparing for the Olympics, is still trying to engineer the return of its £8,000 (US$16,000) meteorological station, which the Chinese government seized in late 2006. Sometimes, as in Arrowsmith's case, the penalty fell on the Chinese collaborator, who received a fine. Others contacted by *Nature* did not want to discuss their situation lest it affect the fate of future applications to do research in China.

The new laws require long, detailed demonstrations of an 'equitable partnership' in which data will be jointly gathered and interpreted with Chinese researchers. The proposals must also describe why the project is in China's best interest, says Xu, adding that some projects, such as those fully funded by a foreign agency, are unlikely to get approved. "International collaborations will definitely drop in number," he says.

The US National Science Foundation's Beijing representative William Chang says that the organization is trying to spread the word to its new grantees working in China about longer application times and higher overheads to cover a greater number of Chinese researchers. "Scientists need to be better coordinated with their Chinese colleagues and better organized."

Arrowsmith is still optimistic that his project-extension request will be approved, although he says that he may have to bring a meteorological-administration official with him. ■

David Cyranoski

J. R. ARROWSMITH

China is clamping down on unauthorized use of GPS and meteorological surveying tools by foreigners.

SPECIAL REPORT

An indifference to boundaries

As some of the world's largest universities undergo dramatic departmental restructuring to foster interdisciplinary research, **John Whitfield** asks whether they're making the right move.

Immunologists at Imperial College London have been tripping over a sticky problem: the structures of the molecules they are working on. The obvious go-to team is the institute's strong corps of structural biologists. But the immunologists are in the division for cellular and molecular biology, whereas the structural biologists are in the division of molecular bioscience. Splitting the funding — and the credit — causes turf wars. The solution? A department of life sciences that merges three biological divisions. "We decided we needed to break the incentive to be selfish," says ecologist Ian Owens, who heads the new interdisciplinary department.

Established three months ago, the department is part of a trend at traditionally structured universities towards initiatives that foster interdisciplinary research. Harvard University — which has a reputation as a place of powerful departmental fiefdoms — and University College London are also rejigging their institutions to remove internal barriers and encourage researchers to come together in new combinations. Part of the trend springs from subject areas that have emerged over the past decade — such as global health, climate change, neuroscience and systems biology — that straddle the boundaries of older disciplines.

It is an idea pioneered by boutique institutes such as Santa Fe Institute in New Mexico, where complexity theory was developed; Bell Laboratories in New Jersey, where lasers and information theory were developed; and the UK Medical Research Council's Laboratory of Molecular Biology in Cambridge, where a group of physicists-turned-biologists pioneered molecular biology.

Social engineering

"Without any exceptions, over the past century the lead scientist on any major discovery has internalized a great deal of scientific diversity," says science historian Rogers Hollingsworth of the University of Wisconsin-Madison. He studies the types of research that lead to major breakthroughs in biomedical science — the kinds that win Nobel, Lasker or Crafoord prizes — and what gives the places that do that research their edge. Such internalization, Hollingsworth says, is most likely to happen in small institutes that have few internal barriers and flat hierarchies, where the bosses stay close to the labs. He points to Rockefeller University in New York and the California Institute of Technology in Pasadena as the exemplars of such an ethos.

UCL

Getting the right people together is important (see 'So, you want to be interdisciplinary …'), as is the physical environment. While planning for the Janelia Farm research campus in Loudoun County, Virginia, director Gerald Rubin discovered that many of the most successful research institutes valued their canteen above all other facilities, owing to the contacts it helped people to create. So Janelia Farm serves three meals a day, seven days a week — but the cafeteria is open for just 90 minutes at lunchtime, encouraging people to bump into one another. Tables seat eight people, but research groups have a maximum size of six, so they must mingle, and you pay more for take-out than eating in. There's also an on-campus pub, serving free coffee all day to deter people from brewing up in their labs, and beer and meals in the evening. "We have done a huge amount of social engineering," says Rubin.

But while distinct departments still control rewards and credentials, not everyone believes that traditional universities will achieve the interdisciplinary success of specialist institutes such as Santa Fe — or even that it is a worthwhile exercise to attempt.

"Interdisciplinary is becoming the buzzword in science, but I'm extraordinarily sceptical about what's going to result in the next 10–15 years from this," says Hollingsworth. "Large research organizations have an enormous

Escape the intellectual blinkers

Researchers working in traditional departments have several places to go to escape the grind of teaching, applying for grants and running a lab. And the demand for such places is rising, says ecologist Marten Scheffer of Wageningen University in the Netherlands, who is helping to set up two interdisciplinary institutes.

Scheffer is a founding father of the Institute Para Limes (IPL), currently being installed in a fourteenth-century monastery in Doesburg in the Netherlands. The IPL started running scientific meetings last year and plans to be fully operational by 2012, with an annual budget of €5.5 million. It will be staffed by cast of 'visitors' coming in for anything from a few days to a few months, and what they do is up for grabs, says Scheffer. "The most important thing is to bring the right mix of people together, and let it evolve."

He is also involved in setting up a similar but more focused institute in Uruguay, the South American Institute for Resilience and Sustainability Studies, which will look at issues such as fisheries, biodiversity and climate change from the broadest possible viewpoint. Like the IPL, everyone will be just visiting. Meetings should start in 2009. "We're planning to get policymakers involved at an early phase," Scheffer says. He hopes that humanities researchers, politicians and artists will also visit the institute. **J.W.**

GIANT FROG FOUND IN MADAGASCAR
'Frog from hell' fossil hints at later split of continents.
www.nature.com/news

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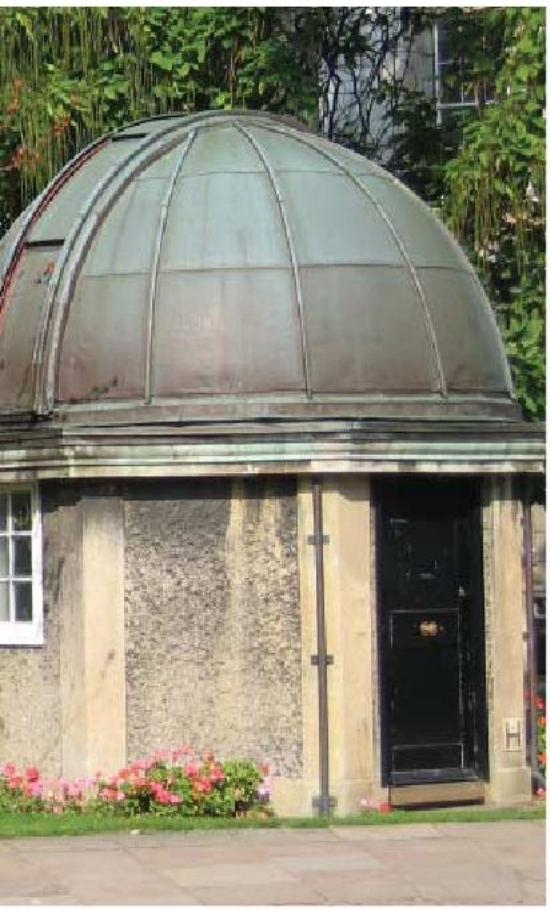

A. CAMPBELL; P. FETTERS/HHMI

Traditional universities such as University College London (left) are restructuring to encourage interdisciplinary research, inspired by the purpose-built Janelia Farm Research Center (top). Collaborations are fostered at canteens (Santa Fe, middle) and in the bar (Janelia Farm, bottom).

amount of inertia, and individuals have a great vested interest in the way they were trained, and what they were doing yesterday."

"It's the Walmart model of the university," complains pharmacologist David Colquhoun of University College London, who is unhappy that his department has become part of a new faculty of life sciences. "There's never been any barrier to interdisciplinary work — you can just pick up the phone or e-mail."

Publishing problems

But neuroscientist Paul Grobstein, who ran an interdisciplinary centre at Bryn Mawr College in Pennsylvania, says that traditional structures make it hard for researchers to be interdisciplinary. "Younger faculty tend to be concerned that if they get involved [in interdisciplinary work], their colleagues in the departments in charge of promotion and tenure will feel they haven't lived up to the standards of the discipline." Other problems, he says, include finding places to publish — "it's much easier for people to get published in traditional disciplinary settings" — and finding an audience. A physicist could, say, publish a paper on stock-market patterns in *Physical Review E*, but how many economists will read it is another matter.

Such problems will be difficult to address through restructuring of traditional universities. Even advocates of interdisciplinary research think that the traditional departmental model will, and should continue to be, used in the majority of cases. It is needed, for example, to support undergraduate teaching and create excellence in specialist subjects. "The drive to form disciplines is a very reasonable one," says Sean Eddy, a computational biologist working at Janelia Farm. "It's phenomenal to be part of a group of labs all thinking the same thing." But you need the alternative, he says: "There's a normal mode of science that works very well, that I wouldn't want to change. But when you're trying to crack something really new, you need people with different experiences to work together."

Rather, Hollingsworth says, the solution may be to spend a small proportion of the national research budget on many small institutions in which scientists can work with as much autonomy as possible (see 'Escape the intellectual blinkers'), Hollingsworth says. "It's easier to establish a new research organization than it is to change an older one." ■

So, you want to be interdisciplinary ...

Interdisciplinary research is not for everyone, and personality is hugely important. At the Santa Fe Institute in New Mexico, president Geoffrey West is always looking for people who have the right mindset. "You need a person with a passion for a bigger picture of science, who can see beyond boundaries and wants to see where the threads of their ideas might lead in other contexts." But, he adds, philosophy does not guarantee quality. "There are extraordinarily smart and creative people that don't care about anything outside their discipline. And there are flaky people who are interested in everything at a very superficial level."

Here are some tips:

Pay your dues Traditional disciplines give you a strong base from which to launch yourself. "If you're not well educated in a basic discipline you can't do interdisciplinary research," says Kathleen Buckley, director of academic affairs for interdisciplinary science at Harvard University in Cambridge, Massachusetts.

Listen — and explain "Traditional disciplines have very different cultures, languages, criteria for judging what's good, and even senses of what science is," says West. "It's very easy to look over at another discipline and say 'that's a bunch of rubbish' — and it's important to make sure that doesn't happen."

Be humble Meetings of minds don't work if one party does all the talking, says Marten Scheffer from Wageningen University in the Netherlands. "Having alpha-male scientists at interdisciplinary institutes is a risk," he says. "If you have one or two very dominant people it can destroy openness."

Be patient Sean Eddy of Janelia Farm in Virginia started his research career as a developmental neurobiologist. He's now a computational biologist, but it's taken him until his early 40s to learn the requisite computer science, maths and statistics. "It was slow and painful," he says. "It's only just now that I feel I'm trained enough across three or four fields that I can get something done."

Be brave Exploring new ground is risky, says Janelia Farm director Gerald Rubin. "This isn't a place for every scientist. You need a large amount of self-confidence and the willingness to take risks. We say: 'We're going to bet $10 million, and you're going to bet your career'." **J.W.**

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NW125311R

Florey Neuroscience Institutes

DIRECTOR

The Florey Neuroscience Institutes (FNI), a substantial neuroscience institute of international significance, is seeking to appoint an inspirational leader as Director due to the impending retirement of the Foundation Director, Professor Frederick Mendelsohn AO FAA. The FNI is located in Melbourne at two sites, the University of Melbourne (Parkville) and Austin Health (Heidelberg), both of which are renowned centers for medical research. The appointee will possess an international reputation as a researcher and research manager, commanding the respect of peers.

The FNI was created by the recent amalgamation of the Howard Florey Institute, the Brain Research Institute and the National Stroke Research Institute. Capitalizing on this unique convergence of talent in neuroscience, the new Director will have the opportunity to lead the next phase of growth, enhancing the Institute's reputation as a leading international research organisation and for nurturing new scientific talent. This will require the further development of strategic relationships, collaborative partnerships and commercial ventures.

For a confidential discussion, to find out more about this challenging leadership role and to acquire an information package including selection criteria contact Dr Rachel Lucas or Ms Evie Watt at Heidrick & Struggles, on +61 2 8205 2000.

Applications including curriculum vitae should be sent in confidence to Dr Rachel Lucas at Heidrick & Struggles, Level 28, 1 Farrer Place, Sydney, NSW, 2000, Australia, or email: fnidirector@heidrick.com by 6 March 2008.

For further information on the FNI visit the following website: www.florey.edu.au/about/the-florey/florey-neuroscience-institutes/

JP124435R

The University of Massachusetts Medical School (UMMS) is seeking a senior leader to direct a new program in Stem Cell Biology

The program will have state-of-the art research facilities, ample space and funding to support new tenure track faculty including a Chief Scientist who will oversee the operation of the International Stem Cell Registry and Human Embryonic Stem Cell Bank. The Stem Cell Program expects to work closely with new programs in RNAi Therapeutics and Gene Therapy. Together, these programs will be an important component of UMMS commitment to clinical and translational science.

Successful applicants will be leaders in their field with internationally recognized research programs, and have the vision and ability to build the program, and recruit outstanding and diverse faculty. The program will play a pivotal role in the development of UMMS Advanced Therapeutic Cluster (ATC), a key element in the Commonwealth of Massachusetts' Life Science Initiative.

As an equal opportunity and affirmative action employer, UMMS recognizes the power of a diverse community and encourages applicants from individuals with varied experiences, perspectives, and backgrounds.

Applicants should send a curriculum vitae and cover letter electronically, briefly outlining their interest in the position to:
gary.stein@umassmed.edu
Dr. Gary S. Stein
Chair, Search Committee
Department of Cell Biology
University of Massachusetts Medical School
55 Lake Ave. North
Worcester, MA 01655

NW124580R

Scientists urged to plan for the next US president

Quit whining and get proactive. That's the message that policy experts gave scientists at the weekend. They advised them to stop complaining that politicians don't take science seriously and instead prepare for the presidential changeover next January.

Scientists were urged to organize now — for instance, by coming up with a list of names for high-profile science positions in the new administration, no matter who runs it.

There are just 77 days between the 4 November presidential election and the 20 January 2009 inauguration, notes Neal Lane, a senior fellow at the James A. Baker III Institute for Public Policy at Rice University in Houston, Texas, who served as science adviser to President Bill Clinton. During those 77 days, the entire executive branch of the government must be set up, including some 50 positions particularly important to science and technology. "It's a huge undertaking," Lane says.

J. REED/REUTERS

Don't wait: scientists told to start lobbying presidential candidates such as Barack Obama (left) and Hillary Clinton.

Lane and other experts were addressing researchers at the annual meeting of the American Association for the Advancement of Science (AAAS) in Boston, Massachusetts. The hotly contested presidential race dominated much of the meeting: in the hallways, advocates passed out lapel buttons promoting the idea of a science debate between the candidates (see *Nature* **451**, 621; 2008). The Union of Concerned Scientists, an advocacy group, announced a "call to action for scientific freedom and the public good", asking Congress and the White House to support integrity in federal science. And representatives of Democratic presidential candidates Barack Obama and Hillary Clinton spoke to a packed room about the positions their candidates hold on science and technology.

Underlying this activity is a palpable frustration with eight years of President George W. Bush's administration, during which scientists have spoken out about the perceived manipulation of science for political ends (see *Nature* **427**, 663; 2004). Policy experts advised researchers to waste no time in trying to change that. Lane, for instance, said the community needs to line up the names of top scientists who could serve as science adviser, as well as lobby to restore the title of 'special assistant to the president' — a direct reporting line to the president that was removed by Bush. Other key positions to be filled include possible heads of the National Science Foundation, the National Institutes of Health and NASA.

At the conference, representatives from the Obama and Clinton campaigns competed to win the hearts of the assembled scientists. Clinton adviser Tom Kalil of the University of California, Berkeley, ran through key points in Clinton's 'innovation agenda', which includes doubling basic research funding over 10 years. Alec Ross, a technology entrepreneur and adviser to Obama, focused on his candidate's plans to expand broadband and technology infrastructure to all Americans. He also hinted that within weeks Obama would unveil a new plan for NASA and space exploration.

> "No matter who wins, we'll be light years ahead of where we are."

Both advisers said their candidates were considering participating in the science debate, planned for 18 April at the Franklin Institute in Philadelphia, Pennsylvania. Invitations have also gone to John McCain and Mike Huckabee. The pair, together with Ron Paul, were all invited to the AAAS meeting, although none sent a representative.

Outgoing AAAS president David Baltimore says he sees little difference between the Democratic candidates, and knows little about where McCain stands on science issues. But no matter who wins, he says, "we'll be light years ahead of where we are". ■

Alexandra Witze

ON THE RECORD

"Allowing an 8-year-old elephant to conceive is the equivalent of allowing your 12-year-old daughter to become pregnant."

A spokesperson for the International Fund for Animal Welfare reacts to news that Thong Dee, who lives at Sydney's Taronga Zoo, has grown up rather too quickly.

SCORECARD

Space veg
Kimchi, South Korea's pickled-cabbage-based treat, has been cleared for launch on the nation's first space expedition this April — minus a bacterium usually used in the recipe that could pose health problems in orbit.

Bad brew
Scientists who like a few beers publish fewer papers and get their work cited less often than their more abstemious colleagues, according to Czech research.

NUMBER CRUNCH

9 shark species are set to be added to the World Conservation Union's list of endangered species this year.

1 fatal shark attack took place worldwide in 2007 — a 20-year low.

33 years ago, Steven Spielberg released *Jaws*, adding to sharks' fearsome reputation and, in the eyes of some conservationists, worsening their plight.

SHOWBIZ NEWS

Seal appeal
Former Baywatch babe Pamela Anderson has used her publicity-courting skills to speak out against Canada's culling of baby seals. "It sickens me, not just as a Canadian but as a human being," she explains.

E. RYAN/GETTY IMAGES

Sources: The Times, BBC, Null Hypothesis, The Guardian, PhysOrg.com, Reuters

SIDELINES

On the origin of deleterious mutations

A genetic study into Americans of European or African descent finds that the Europeans have a bigger proportion of 'harmful' genes than the Africans. But the conclusion has already been questioned in one of what many expect to be a fresh wave of conflicts over data interpretation as new technologies enable a glut of population-genetics studies.

D. TURNLEY/CORBIS

Controversy over data interpretation is likely to increase with the glut of population-genetics studies.

Carlos Bustamante, a statistical geneticist at Cornell University in Ithaca, New York, and his colleagues compared genetic variation in 20 European Americans and 15 African Americans (K. E. Lohmueller *et al. Nature* 451, 994–997; 2008), looking specifically at single nucleotide polymorphisms (SNPs) — places where DNA differs between individuals by just one 'letter' of the genetic code. The researchers provide the first unbiased genomewide count of these mutations (previous data sets have not agreed on this number) — it is a significant advance in the field of population genetics.

The findings confirm the idea that after human groups migrated out of Africa, they experienced a population 'bottleneck' in which their overall genetic diversity was reduced. The European population then expanded quickly, accruing new mutations before there was time for the old ones that caused negative consequences to be weeded out. The researchers built computer models to show that their data fit this bottleneck interpretation.

The result is that the European population contains a lower overall genetic diversity than the African population. And a higher proportion of the European genetic diversity is potentially harmful, as the DNA contains mutations that could alter the function of the proteins it encodes, the team says.

However, Alexey Kondrashov, a population geneticist at the University of Michigan in Ann Arbor, says that the data could be interpreted differently — he says that the African and European populations have exactly the same burden of harmful traits.

> "It is difficult to say how single nucleotide polymorphisms would affect a person's health."

It all boils down to a small difference in interpretation of the data. The researchers analysed 39,440 SNPs, looking at each one to see whether it matched the ancestral SNP — the one found in the genome of the chimpanzee, the closest genetic relative to humans. The team then examined those found to be non-ancestral — called derived SNPs — to determine whether the change had affected the identity of the amino acids (each made of up of three DNA letters) they encode. The 'non-synonymous' SNPs, the derived SNPs that encoded a different amino acid from the ancestral SNP, were classified by the team as more likely to be damaging. Software was used to classify SNPs as either 'benign', 'possibly damaging' or 'probably damaging' to the proteins they encode.

The team then examined the SNPs unique to each population and found that 47.0% of the African Americans' SNPs were non-synonymous, compared with 55.4% of European Americans' SNPs. Of these non-synonymous SNPs, 12.1% were in the 'probably' damaging bracket for African Americans, compared with 15.9% in European Americans — a small, but statistically significant difference. "The interesting finding in our paper is that, given lower levels of variation on the whole in Europe than in Africa, a disproportionate amount of [the European variation] is deleterious," says Kirk Lohmueller, a statistical geneticist at Cornell.

However, Kondrashov points out that Bustamante's group also counted the number of non-synonymous SNPs, as well as the 'possibly' and 'probably' damaging SNPs, in the Americans. They found that the European population had about the same number of non-synonymous, possibly-damaging and probably-damaging SNPs as the African American population. These data, as presented in the paper (see Fig. 1, page 995) should be interpreted differently, according to Kondrashov. It indicates that the two populations are carrying the same number of potentially deleterious SNPs, he says. "The title of the paper is misleading because they say they showed that Europeans are more genetically burdened than Africans," he says. "But this is simply not true, if you look at Figure 1."

Bustamante disagrees, pointing out that the figure was intended to summarize the genotypes of the individuals in the study — that is, the catalogue of which two SNPs are carried on a person's chromosomes. And, Bustamante says, it is difficult to say how these SNPs would affect a person's health when removed from their genotypic context, so it is not entirely correct to use them as a basis for comparing the overall fitness of the two populations. "We don't interpret Figure 1 to show that individuals in one population or the other are more fit or are more healthy," says Lohmueller.

"We are struggling with the gulf between these statistical descriptions of whole populations and their implications for individual risk," says Andy Clark, a Cornell statistician and one of Bustamante's coauthors. "Just because these patterns exist does not mean any European American individual is going to be at any more risk from disease than any African American." Driving that point home is going to become increasingly difficult — but extremely important — as more population-genetics studies pour out over the coming year. ■

Erika Check-Hayden

NOAA

ARCTIC MAPPING REDRAWS BORDERS
Secrets emerge from 'least-mapped place in the world'.
www.nature.com/news

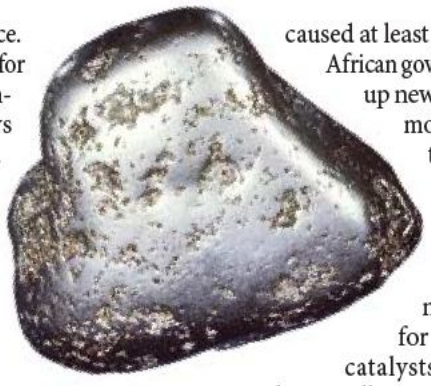
Energy crisis upsets platinum market

Electricity shortages in South Africa led to record-breaking prices for platinum last week as two of the world's leading producers forecasted that energy rationing will reduce production in 2008.

South Africa has been plagued by energy problems for months, but major blackouts lasting up to several hours started sweeping the country in January. Eskom, the state-owned utility that provides most of the nation's electricity, has said that it will cut supplies to industrial customers by 10% to help stabilize the situation.

Anglo Platinum and Implats, the world's largest platinum producers, say that the power shortages could result in less production of the metal in 2008. The price of platinum, which is mostly used in catalytic converters for vehicles, breached US$2,000 per troy ounce for the first time ever on 14 February; a month earlier the price was just under $1,600 per troy ounce.

Jeremy Coombes, an analyst for the catalyst manufacturer Johnson Matthey in London, says that the forecasts amount to a 3–5% decrease in production — big numbers for a market that is already tight. "The market was very sensitive and it really got hammered by the news about the power shortages," Coombes says.



E. R. DEGGINGER/SPL

"The platinum market got hammered by the news about the power shortages."

Eskom says that the situation might not return to normal until 2012, when it expects to start up at least one new power plant. This expansion of power should have started years ago, but the company's plans have been repeatedly delayed, according to Anton Eberhard, an energy expert at the University of Cape Town. Eberhard believes that the problem was caused at least partly by the South African government, which held up new projects while promoting privatization in the energy sector.

South Africa produces roughly four-fifths of the world's new platinum. Rising demand for the metal in autocatalysts — used to lower exhaust pollution — has been driving prices up since 2002, when platinum sold for as little as $451 per troy ounce.

Coombes says that the price increase will probably spur further efforts to recycle the metal from autocatalysts, an activity that provided 11% of the global supply in 2006. It could also wipe out the market for platinum in all but the most expensive jewellery. ■

Jeff Tollefson

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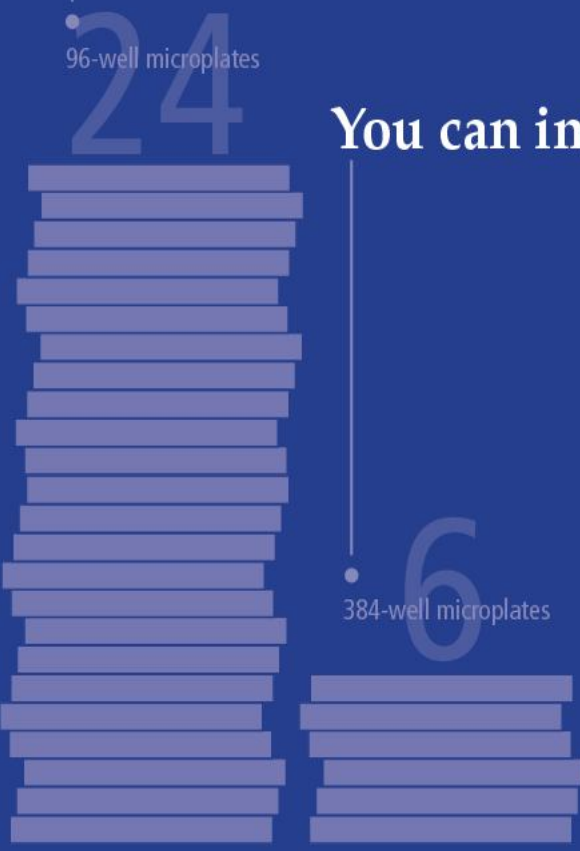

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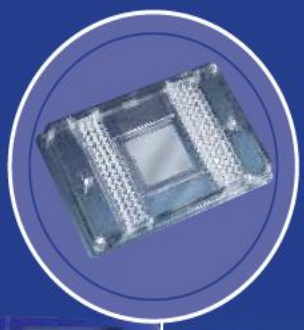

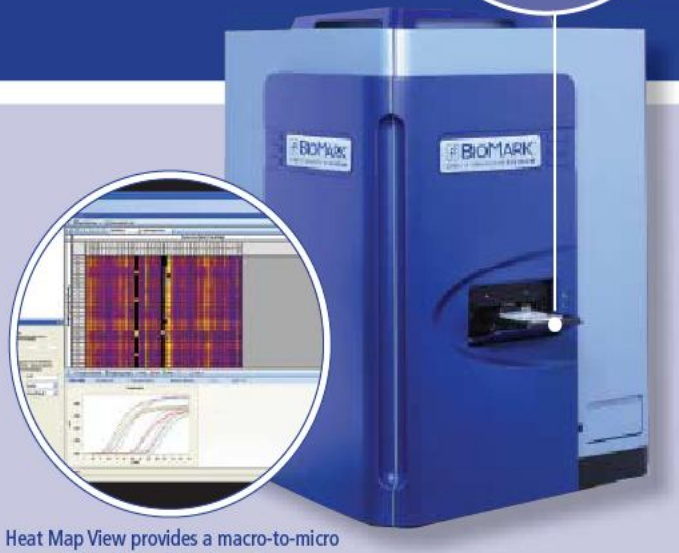

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No jail for geneticist who posted bacteria to artist

A researcher at the University of Pittsburgh, Pennsylvania, last week escaped a jail sentence but was fined US$500 for sending bacterial samples to a performance artist in Buffalo, New York.

In 2004, geneticist Robert Ferrell supplied Steven Kurtz, an art professor at the State University of New York at Buffalo, with samples of *Bacillus globigii*, *Serratia marcescens* and a benign form of *Escherichia coli*. Kurtz used bacteria in experiments involving the audience to explore attitudes towards bioterror and genetic engineering in shows by his performance art troupe, the Critical Art Ensemble.

Kurtz's wife suffered a fatal heart attack in May of that year. Emergency workers who arrived at the artist's home became suspicious when they found the samples and laboratory equipment (see *Nature* 429, 690; 2004). Federal prosecutors later charged Ferrell and Kurtz with mail and wire fraud.

Last October, 64-year-old Ferrell pleaded guilty to one count of mailing an injurious article. He describes the prosecution as a "nuisance". Kurtz still faces prosecution and is fighting to have the case thrown out.

Funds run dry for sea-fertilization project

Ocean-fertilization company Planktos has indefinitely suspended its plans for large-scale dumping of iron powder in the ocean. The company claimed that such a project would have seeded phytoplankton blooms to sequester carbon from the atmosphere. Eventually, Planktos said, the phytoplankton would sink to the ocean floor, taking their carbon with them.

"A highly effective disinformation campaign waged by anti-offset crusaders" made raising the required funds difficult, the company based in Foster City, California, stated last week.

But critics say that more research is needed to understand the possible environmental effects of such large-scale iron releases, including hypoxia and the production of other greenhouse gases.

PLANKTOS

Planktos has halted its plans for iron fertilization.

Harvard adopts opt-out open-access policy

K. SNIBBE/HARVARD UNIV.

Harvard University (pictured) has adopted guidelines under which the 'final drafts' of academic papers written by researchers at its Faculty of Arts and Sciences will automatically be published on the university's website, unless the authors request a waiver. Immediate open access to papers could conflict with the copyright policies of many journals including *Cell*, *Nature* and *Science*.

Many institutions keep open-access repositories of papers but the decision makes Harvard the first US university to sign up to default open-access publishing for its research staff. Although the University of California has toyed with the idea for years, it has yet to agree on a policy.

Stuart Shieber, the computer scientist at Harvard who proposed the scheme, says that any request for an exemption will be granted. The university has not yet worked out how to define what constitutes a 'final' draft of a scholarly paper, nor come up with a time limit for submission.

Critics of open-access policies worry that highly selective journals with large readerships will suffer, and that non-peer-reviewed research will become more prominent.

Systems biologists hatch plan for virtual human

Researchers in the fledgling field of systems biology have laid down the challenge of creating a molecule-based computational model of a person that could be of use to the pharmaceutical industry.

The 'virtual human' would simulate the interactions between the tens of thousands of human proteins and other cellular components, such as non-coding RNA. Researchers agreed to try to create such a model within the next 30 years at a three-day workshop in Tokyo earlier this month.

If the plan goes ahead, it would require the development of new technologies and collaborations between researchers from different countries and disciplines, says Hiroaki Kitano, director of the Systems Biology Institute in Tokyo. Kitano hopes that the ambitious goal will help win support from Japanese and UK funding bodies for research into systems biology.

India has a key satellite antenna stolen for scrap

A crucial Global Positioning System (GPS) antenna in Bangalore has been stolen — apparently for its scrap value — knocking India out of an international network of 'core' stations that provides data to geoscientists around the globe.

The station at the Indian Institute of Science was linked to the International Global Navigation Satellite Systems Service, based in Pasadena, California. The service provides scientific data such as for satellite navigation and earthquake-risk monitoring. Although India has 2 of the 336 active stations in the global network, the Bangalore station was the only one among the 40 core stations that supply data in real time.

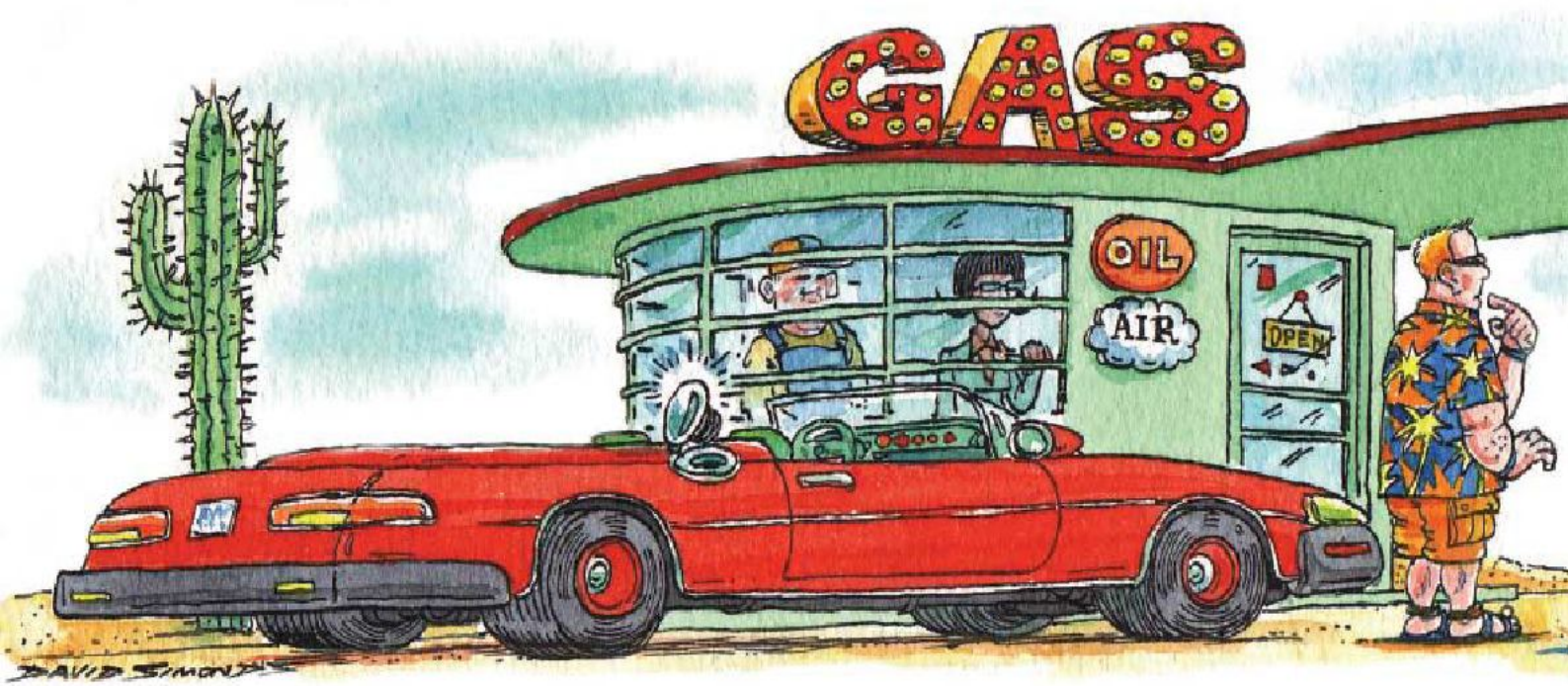
The loss means that the "global Earth-science community will not have real-time GPS data from the Indian subcontinent", says Sridevi Jade a geoscientist at Bangalore's Centre for Mathematical Modelling and Computer Simulation, which maintains the station. She says that it will take a year to erect a new antenna in the same location and make it operational.

Medical schools still not declaring financial conflicts

Many US medical schools do not have policies to address potential conflicts of interest, according to a study published last week (S. H. Ehringhaus *et al. J. Am. Med. Assoc.* 299, 665–671; 2008).

In a survey by Susan Ehringhaus, associate general counsel at the Association of American Medical Colleges, and her colleagues, just 38% of the 86 medical schools that responded reported having a policy in place that covers their financial interests, such as royalties and stocks. Another 37% of schools said that they were working on developing a policy.

The numbers rose markedly when the schools were asked whether they had policies to cover individuals: 71% of the schools had policies for senior officials and 66% had them in place for governing board members.



Not your father's biofuels

If biofuels are to help the fight against climate change, they have to be made from more appropriate materials and in better ways. **Jeff Tollefson** asks what innovation can do to improve the outlook.

Biotechnology has changed the way that drugs are discovered, designed and, often, made. It has spread new capabilities across the farms of much of the world, sometimes amid much controversy. Now some of its advocates are suggesting that it is poised to overhaul the energy sector as well, changing both the crops that are grown and the fuels that are made from them.

Entrepreneurs have attracted hundreds of millions of dollars for bio-energy companies working on 'second-generation' fuels produced from crop residues, grasses or woody materials that avoid the shortcomings of ethanol distilled from corn starch or biodiesel produced from oil crops. Some are offering hope for higher yields from less land, from more marginal land, and with less investment in terms of energy and fertilizer; others are promising fuels better suited to the needs of drivers and the existing fuel infrastructure. Even the major oil companies are getting involved.

"The market is slated to be so big that there will be opportunities for multiple approaches, and it will probably take many years before we settle on one absolute best approach," says Doug Cameron, chief science officer for Khosla Ventures, a venture-capital firm in Menlo Park, California, that is backing a large number of start-up bioenergy companies.

Liquid biofuels will never make up a significant portion of the global transportation fuel supply unless biologists and engineers make the most of these opportunities. During the past four years alone, global ethanol production has more than doubled to nearly 50 billion litres (about 13.2 billion gallons) in 2007. Biodiesel, although starting out much lower, nearly quintupled to 9 billion litres (2.4 billion gallons) during the same period. This rate of growth is not sustainable — and with current production methods far from desirable, because the agricultural techniques used often damage the environment on a scale that far outweighs any good achieved through the biofuels' use.

> "We're not screwing around — the basic science is done."
> — Pat Gruber

In the United States, which has ramped up production and is now home to the world's largest biofuel industry, roughly 23% of the corn crop goes to ethanol, which in turn provides 3% of the nation's transportation fuels, according to Alex Farrell, an energy and resource scientist at the University of California, Berkeley. Worldwide, biofuels make up less than 1% of transportation fuel, he says. Current technologies could push that as high as 2–3%, "but anything much larger than that will have to be based on significantly different technologies".

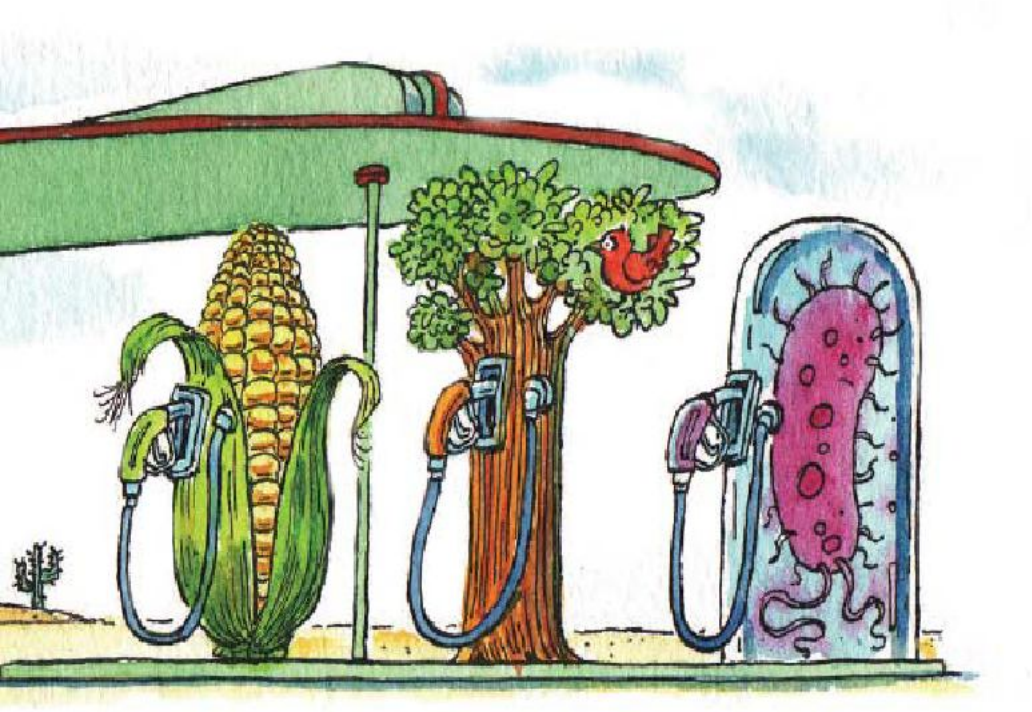
Fuels for the future

Applying biotechnology to biofuels is not a new idea. In 1991 Lonnie Ingram, a microbiologist at the University of Florida, Gainesville, was awarded a patent for an engineered *Escherichia coli* bacterium that converts sugars into ethanol. But only now is the technology really coming into its own — not least because today's 'metabolic engineering' and 'synthetic biology' put much more ambitious fuels than ethanol on the table, or indeed into the pipeline.

"I've always been of the opinion that ethanol is for drinking, not driving," says Jay Keasling, a chemical engineering professor at the University of California, Berkeley, who has pioneered the synthetic biology needed to get microbes to produce various new classes of molecule. To take one example: drinking is made easy by the fact that ethanol and water mix easily. That's good for scotch and soda, but bad for pipelines, which give the fuel a chance to get watered down and contaminated. In the United States, home to the largest biofuels industry in the world, trucks or train cars carry the fuel from the agricultural heartland where corn is grown to the coastal areas where fuel is needed most. In Brazil, the other biofuel giant, where sugar-cane ethanol is produced much more efficiently, the agricultural source and the urban users are relatively close together in the country's southeast — one of the many advantages the Brazilian industry enjoys.

This is why Amyris Biotechnologies, a start-up company in Emeryville, California, of which Keasling was a co-founder, and a number of other small companies in California and Massachusetts are designing microbes to make better fuels — fuels with higher energy contents

D. SIMONDS



that are better suited to pipelines and other infrastucture. University researchers are also in the race. Last month Jim Liao, a researcher at the University of California, Los Angeles, described an *E. coli* in which more than a dozen modifications to the metabolic pathways normally used to produce amino acids caused the bacteria to produce isobutanol, an alcohol with four carbon atoms to ethanol's two, and similar molecules. Even without optimizing every step of the process, Liao's lab was able to achieve a yield of 86% of the theoretical maximum (S. Atsumi, T. Hanai & J. C. Liao *Nature* **451**, 86–89; 2008).

His process has been licensed by Gevo in Pasadena, California, a start-up company funded by Khosla Ventures, among others, which says that it hopes to begin commercial-scale production within a few years. Gevo chief executive Pat Gruber says that the technology could be retrofitted on an existing bioethanol plant for as little as US$20 million: "We're not screwing around — the basic science is done. We are going to try and get this stuff developed and into the marketplace."

Another form of butanol has attracted oil-giant BP and DuPont, one of the world's largest chemical companies, which are working together on biofuels. Butanol has a higher energy density than ethanol, offering roughly 85% of the energy content of a standard petrol mix, compared with about 66% for ethanol; this offsets the fact that less butanol than ethanol can be made from a given amount of biomass (there are only so many carbon atoms to go round). Although other molecules could pack even more energy, DuPont officials say that they settled on butanol as a molecule that meets their needs and can be developed quickly.

DuPont has a track record in the sort of metabolic re-engineering required for such things: the *E. coli* that churn out propanediol, a chemical used in various materials and industrial processes, in its facility in Loudon, Tennessee, have had 30 changes made to their metabolic pathways. It took about 11 years for that project to get from the proof of concept to production, but officials say biobutanol could be sorted out much faster. "In the old days, it would take four to six months to clone a new gene," says John Pierce, DuPont's vice-president for applied biosciences. "Now it takes two weeks and you usually do it by mail."

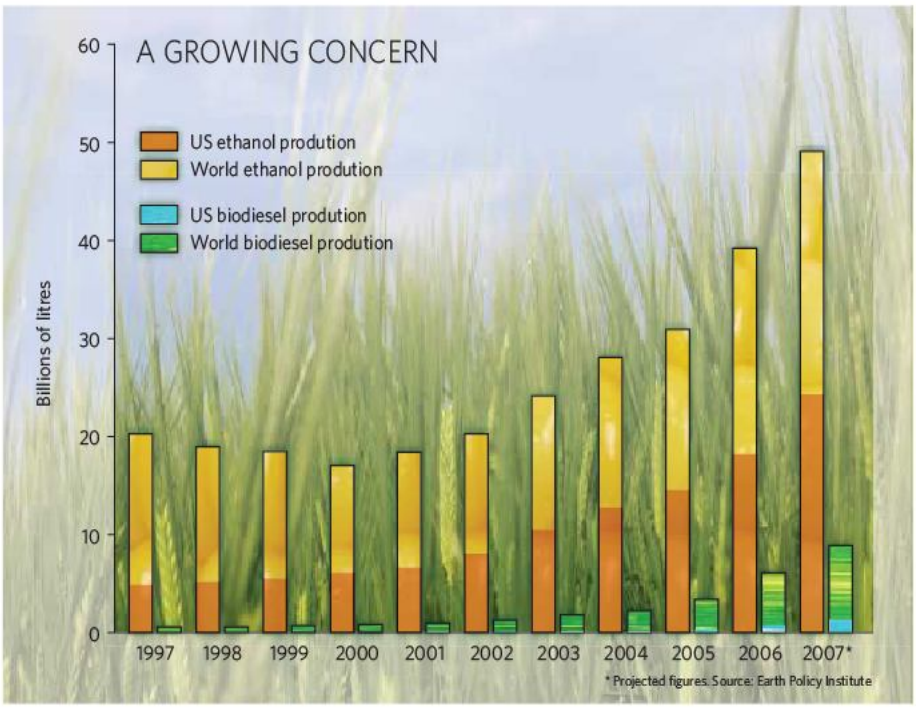
Butanol's advocates say that their fuel could be run through a refinery to produce longer chain molecules as desired. Amyris and LS9 of Cambridge, Massachusetts, are looking to skip this step and move straight to molecules more like those that engines already burn. Both companies specialize in synthetic biology — which aims to create new biological entities in a much more thoroughly designed way than traditional genetic engineering — and both have received backing from Khosla Ventures, among others.

LS9 was co-founded by George Church, a geneticist at Harvard Medical School in Boston, Massachusetts, and Chris Somerville, a plant biologist at Stanford University in California, who previously headed the Carnegie Institution's department of Plant Biology. Somerville is currently heading the new Energy Biosciences Institute (EBI), a partnership between the University of California, Berkeley, Lawrence Berkeley National Laboratory and the University of Illinois at Urbana-Champaign. The EBI was kick-started in 2006 with a $500 million pledge from BP.

Different pathways

LS9 wants to transform the fatty acids naturally produced by *E. coli* into specific hydrocarbon fuels; it has announced plans for a pilot plant and says that commercial production could begin within two to three years. Amyris decided to take yeast, which is known for its ability to make ethanol, and train it to produce longer-chain molecules such as gasoline, diesel and jet fuel.

The company has engineered roughly 1,000 strains of yeast so far and expects to build as many as 2,000 more in the months ahead. When a strain looks promising, the scale-up begins, as molecular biologists hand their product to chemical engineers and fermentation engineers. In addition to producing exactly



what is needed, Jack Newman, Amyris's senior vice-president for research, says that the technology could prove to be extremely versatile. "You could have your plant making diesel fuel one day and almost literally turn around and make jet fuel the next."

Wood pulp and fiction

All these companies are looking at traditional raw materials for their wonderbugs — sugars. And this means that although they may make more attractive and easily distributed fuels, they will not of themselves change the industry's productivity. To do that requires cheaper and more sustainable feedstocks than sugars and starches from cane and corn.

"I'm of the opinion that the crucial thing that needs to be done is not actually to make better fuels out of sugars but to make sugars more efficiently from cellulose," says Lee Lynd, a biology and engineering professor at Dartmouth College in Hanover, New Hampshire. "The thing that limits corn ethanol, frankly, is not ethanol — it's corn. Those other molecules will become important in the broader scheme of things if and when we solve the problems with cellulosic biomass."

Turning cellulose — the tough polymer from which the cell walls of plants are made — into biofuel is currently a major focus in the industry. It widens the possible range of feedstocks greatly, making it possible to use crops for biofuels that are not also food for humans. Legislators are keen to push the industry in that direction. The ethanol mandate enacted by the US Congress in 2007 requires that, of an annual ethanol production of 136 billion litres (36 billion gallons) required by 2022 — more than five times current US production — 44% must come from cellulosic foodstocks, with corn's contribution remaining static from the mid 2010s. Things might be able to go even further. A report by McKinsey & Company, a consultancy firm based in New York, suggests that, at oil prices above $70 per barrel, cellulosic feedstocks could supply half of the global transportation fuels by 2020 (although that figure explicitly ignores many real-world constraints).

The fly in this ointment is that the world cannot yet boast a single commercial-scale cellulosic-ethanol facility. Breaking cellulose down into sugars is not easy work, and can use up a lot of energy; what's more, not all the sugars produced are easily fermented. Despite the recent spike in oil prices on the international market, lenders and investors have hesitated to pump money into commercial-scale ventures, fearing technical risks and a potential drop in the price of oil. To help push the technology over the first economic hurdle, the US Department of Energy (DOE) is pumping $385 million into six demonstration projects. Work begun on the first of them, run by Colorado-based Range Fuels, in Georgia last autumn.

Cellulose can be broken down with heat and catalysts. It can also be broken down with biology. This is where biotechnologies can come in. Dartmouth's Lynd is the co-founder and chief scientific officer for Mascoma Corporation in Cambridge, Massachusetts, which is pursuing work on a bacterium that can produce the enzymes to break down cellulose on demand as part of the process. Verenium Corporation, also in Cambridge, the first publicly held cellulosic-ethanol enterprise, is developing both new enzymes and organisms to break down the structural tissue. Other companies and researchers are looking to the microbes that break down cellulose in the guts of cows and termites, or to fungi — that is to say, the natural world's most accomplished consumers of the cellulose found in grasses and wood.

"I've always been of the opinion that ethanol is for drinking, not driving." — Jay Keasling

Finding a feedstock

If cellulosic technologies can be made to work, there's still the question of where the cellulose comes from. For the past ten thousand years, most human meddling with the proclivities of plants has been designed to make them better to eat — more sugar in the cane, more starch in the corn, more protein. Energy crops, though, require a different approach. No need for fruit or seed — just for fast growing cellulose, ideally in a form that requires little or nothing by way of extra inputs such as fertilizer. Cellulose that is particularly easy to break down gets bonus points.

Switchgrass, a hearty, fast-growing grass native to America's prairies, is one much-touted possibility, but even a brief survey indicates that there could be better options. Steve Long, a professor at the University of Illinois at Urbana-Champaign, has spent years studying *Miscanthus giganteus*, a relative of sugar cane that is native to East Asia. He has studied the grass in Denmark, England and now Illinois, and says that the average yield is double that of switchgrass and 50% higher than corn — without any fertilizers.

According to Long, meeting the ethanol goals laid out in 2006 by President Bush (which were a touch lower than the mandates actually passed by congress) with corn ethanol would require 25% of the nation's cropland. That figure drops to 15% if cellulosic technology allows you to make use of the corn 'stover' — the bits left over after harvest. It drops to 8% if you use *M. giganteus* — and such grasses could be grown on marginal land that is not being used for food production today.

And, according to Long, neither stover nor *M. giganteus* are the last words. "A lot of effort is being invested in modifying corn stover to make it easier for digestion, but I think we need to think much more broadly," he says. "It seems very unlikely that we've come across the very best option."

R. KALTSCHMIDT/LBNLE

Whichever crops look most promising, engineers will be eager to tinker with them. Keasling says that a decade of intensive biotechnology could now do for an energy crop as much as centuries of selective breeding have done for many food crops. New models of cultivation may help too — for example the replacement of monocultures, which are useful if you want a particular form of food, with polycultures that simply maximize biomass. David Tilman and his colleagues, at the University of Minnesota in St Paul, reported last year that plots with a diverse mixture of prairie grasses yielded on average 238% more energy than plots with a single crop (D. Tilman, J. Hill & C. Lehman *Science* **314**, 1598–1600; 2006). All these approaches might be tailored to marginal lands where the soil wouldn't support food crops.

Or you could just do away with soil altogether: that's the appeal of algae. GreenFuel Technologies, based in Cambridge, Massachusetts, is developing algal bioreactors that tap into carbon-dioxide streams from coal plants to produce rich algal crops that can be harvested and turned into biofuels. A more ambitious approach would be to have the algae act as both feedstock and processor, secreting ready-made fuels. Tasios Melis at the University of California, Berkeley, is looking at getting algae to secrete hydrocarbons in a form that can be continuously collected.

Botryococcus braunii, the green alga that Melis is working with, naturally secretes a 30-carbon terpenoid that can be processed into fuel, perhaps in order to reduce the effects of ultraviolet light. Melis says that he has devised a method for collecting the product — a sticky film to which the algae adhere — and is now working to increase production. More ambitiously, various groups, including Melis, are

As it is today: this VeraSun Energy plant near Aurora, South Dakota, produces 450 million litres of corn ethanol a year.

E. LANDWEHR/AP

looking at having algae produce hydrogen.

Another approach, taken by Coskata in Warrenville, Illinois, (yet another Khosla Ventures start-up) and its partner General Motors, is to avoid putting effort into specialist feedstocks and instead develop a process that works universally. Coskata's process converts carbon-based feedstocks, which could be crops, agricultural waste or municipal waste, into carbon monoxide and hydrogen. This is the first step of processes by which coal or natural gas are turned into liquid fuels, a procedure that is normally taken as an alternative to biological means for producing fuels. Coskata says it has a way of combining the two approaches, with microbes that turn the 'synthesis gas' straight into ethanol. The company is currently developing a pilot project that it says will be able to produce ethanol for less than 26 cents per litre ($1 per gallon).

Scaling up

One thing all these innovations share is that they have yet to be attempted on a large scale. Biotech may work well for drug companies that sell small volumes at premium prices, but the economics of biofuels are more in line with the oil and gas industries, which sell in bulk for low prices and are dominated by the cost of raw materials and manufacturing.

One of the problems is that microorganisms evolved to take care of themselves, not people. Re-engineering their metabolisms in such a way that they excrete fuels — which by definition are energy-rich compounds — means convincing them to forgo energy that they might otherwise use to their own ends. Moreover, in many cases the fuels can be toxic to the organisms themselves. All this provides ways for the engineering to come unstuck.

"If [the microbes] are unhappy with what they are doing, they are going to evolve away from what you want them to do," says genome entrepreneur Craig Venter, whose company Synthetic Genomics in La Jolla, California, has an interest in biofuel production. "A key part of the future is going to be designing a system where they are not grossly unhappy with what they are doing." Many researchers see hope in producing longer-chain biofuels precisely because it decreases the stress on the microbes doing the work. Ethanol is poisonous to its producers (which is why fermentation can't on its own produce hard liquor — the yeast dies). Longer-chain molecules will separate out from the medium in which their producers grow; this avoids the costly step of distillation.

Even if the microbial producers can be kept happy, there's a lot more work to do in producing systems that will work on an industrial scale in commercial refineries. "Among scientists it's considered very doable," says Michael Himmel, who heads a team of researchers working on cellulosic ethanol at the DOE's National Renewable Energy Laboratory in Golden, Colorado. "It's difficult work and it's going to take some funding. But it's not cold fusion." But what's doable in principle to a scientist is not always practical to an engineer.

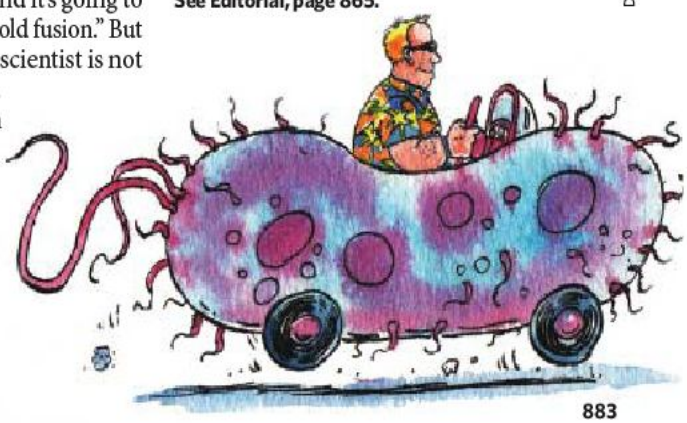
Big oil companies, which would have the know-how for such engineering, are keeping an eye on the field. As well as BP's work with DuPont, Shell is partnering with Iogen in Ottawa, Canada, which ran one of the DOE's cellulosic pilot plants, and with an algal biofuel producer in Hawaii. But all this is small compared to the investments such companies make in their oil-based business.

Biofuels will never take over the whole liquid-fuel market, let alone amount to a large proportion of total energy use. But they, and other technologies, have a part to play. A decade and a half after receiving his patent on an ethanol-producing bacterium, Ingram is still a biofuel enthusiast. But he's also a realist. In the same law that expanded the ethanol mandate, Congress also increased the fuel-efficiency requirements for vehicles by 40%, pushing the average efficiency required by 2020 up to 6.7 litres per hundred kilometres (35 miles per gallon) from the present average of 9.4 litres per hundred kilometres (25 miles per gallon). The technologies to do that are already available — Japan had an average fuel efficiency of 5.1 litres per hundred kilometres in 2002. And as Ingram points out, "If we increase gas mileage by 1 mile per gallon, that is about equal to all the ethanol we are making right now from corn." ■

Jeff Tollefson covers climate, energy and the environment for *Nature*.

See Editorial, page 865.

D. SIMONDS



NATURE|Vol 451|21 February 2008

A FLIGHT TO REMEMBER

The dream of perpetual flight without fuel has inspired pilots to take to the skies in solar-powered planes. **Vicki Cleave** looks at a mission to fly a solar plane through the night — and around the world.

When the Wright brothers made their maiden flight in a powered aircraft on a windswept beach in 1903, it was a short hop, skip and jump into the record books. More than a century later another single-seater aircraft is on its way to making its own record-breaking hops, skips and jumps around the globe. Each of *Solar Impulse*'s wings will cover more distance than Orville Wright's first flight; but the plane's 80-metre wingspan is not what's truly impressive about it. The remarkable thing is where it will get its power — and how little it will need. Driven solely by energy from the Sun, the plane will be carried aloft by solar cells that generate a total of around 9 kilowatts — roughly the same power available to the Wright Flyer from its single engine.

In the Wright era, aircraft were dubbed 'heavier-than-air machines', reflecting the disbelief that they could leave the ground, let alone be successfully piloted. The history of manned solar aviation fosters similar scepticism (see 'Solar aviation highs and lows'). Most solar planes move so slowly through the air, their ungainly frames buffeted by weather, that they challenge our expectations of modern-day flight. Yet the pilots who wish to fly *Solar Impulse* around the world plan on staying aloft for up to five days at a time, and flying through the Sun-starved night.

The US$91-million *Solar Impulse* project is the vision of Bertrand Piccard, a Swiss aeronaut already in the record books as one of the pilots of the first non-stop round-the-world balloon flight in the Breitling Orbiter 3 in 1999. Piccard, who will also be one of the pilots on *Solar Impulse*'s trip around the world, comes from a family of adventurers. His grandfather Auguste made a record-breaking balloon ascent to 23 kilometres in the 1930s and his father Jacques was one of two people to have reached the Challenger Deep in the Mariana Trench, the deepest surveyed point in Earth's oceans.

Piccard says he first thought of the solar project after he stepped out of the Orbiter. "The press was saying that my balloon flight around the world in 1999 was the last adventure that was still possible because everything else had been done," he says. "I was 41 years old at that time, and I thought 'it's a pity if everything has been done.'"

> "I thought it would be great to have a vehicle that would fly day and night with no fuel."
> — Bertrand Piccard

He was also disappointed that the round-the-world balloon trip had used such massive amounts of fuel: having taken off with almost four tonnes of liquid propane, it landed with just 40 kilograms. "We were really limited in fuel, in duration, and if the wind had been slower on the Atlantic we would have ditched and not made it," he says. "I thought it would be great to have a vehicle that would fly day and night with no fuel, with no limit of duration."

Piccard was not the first to be attracted by the notion of fuel-free flight. More than 25 years ago, US aeronautical engineer Paul MacCready from AeroVironment in Monrovia, California, built a plane light enough and slow enough to fly on the low power output of solar cells. His *Gossamer Penguin* weighed less than 31 kilograms without a pilot and its speed over ground was slower than a bicycle. The planned *Solar Impulse* will have a maximum weight of 2,000 kilograms, including the pilot, almost one-quarter of which will be from batteries for storing energy to fly through the night. If all goes well, the plane will fly at speeds of 50–100 kilometres per hour on its round-the-world trip, landing five times along the way to swap pilots.

The trip will be a no-frills experience for the solitary pilot. Inside his snug cockpit, which will protect him from temperature extremes of 80 °C to –60 °C, the pilot will endure the same cramped position for up to five days at a time. "We are not sure how we will sleep or cope with maintaining alertness," says André Borschberg, the other pilot and chief executive of the project.

SOLAR IMPULSE/EPFL CLAUDIO LEONARDI

They will need to be at their most alert when flying the slow-moving craft through the hours of darkness. Although unmanned solar aircraft have made night flights before, no piloted solar plane has stayed aloft for more than 6 hours at a time. The *Solar Impulse* team plans to get through the nights with a mixture of gliding down to lower altitudes and using batteries for power. After dusk, the plane will descend from its maximum daytime altitude of 12 kilometres to just 1 kilometre. The air is denser at lower altitudes, slowing down the plane and reducing the amount of power consumed.

Handle with care

But the biggest challenge will be the weather. Because of its light weight and slow speed, the craft can't handle strong winds or turbulence. "This aircraft is the size of the largest transport aircraft, but it follows any gust that you have," says Borschberg. And despite having a wingspan slightly bigger than that of an Airbus A380, he expects *Solar Impulse* to fly a bit like a hang glider — rather like the Wright Flyer.

When taking off, the pilot can choose the best weather window, but during the crucial overnight descent the plane will be at the mercy of winds and turbulence. "If we have headwinds at night, the night gets longer. If the night is longer the batteries might not be sufficient any more," says Borschberg. Each dawn will look sweeter than the last, as pilot and plane run low on energy.

Fellow solar-aviation experts — many of them pilots themselves — are upbeat about the project's chances. Piccard discussed his plans with MacCready before beginning the project, and his verdict was: "It will take an elegantly crafted vehicle, flown in meteorological conditions that are hard to find, but it's doable." "I'm just very sad he died before we could make our first flight," says Piccard.

MacCready's view is echoed by Chris Kelleher, technical director of the Zephyr project, the record-holder for the longest unmanned solar flight. "It's doable," he agrees. "The issues are the altitudes and the speeds that it would fly at, and the weather conditions." As the on-the-ground pilot for the much smaller Zephyr, Kelleher explains that handling isn't a problem once Zephyr is high enough, above about 18 kilometres. "At altitude, it handles like a big, commercial airliner because the gust sizes tend to be big features, and the aeroplane flies very slowly into the parcels of air."

> "We are not sure how we will sleep or cope with maintaining alertness." — André Borschberg

Because it will be manned, *Solar Impulse* won't be able to fly as high as Zephyr. So Kelleher thinks that weather forecasting will be crucial for achieving the mission. "With stable conditions it's possible to predict the weather and come lower," he says.

But how often will the team be able to count on stable conditions? The planned flight path will follow the Tropic of Cancer, which maximizes the plane's exposure to daylight while hopefully avoiding the worst tropical weather. Borschberg agrees that weather prediction is going to be one of the most important aspects of mission planning. "At take-off it's not too difficult, because you decide when to take off. For landing it's more difficult because you cannot always plan exactly what happens."

To test its weather-prediction systems, the *Solar Impulse* team has been conducting virtual flights since last May. The researchers used a simulator that mimics the performance of the aircraft and allows them to introduce meteorological data. "You can have this aircraft basically flying in real conditions," says Borschberg.

They have learnt some valuable lessons from the simulations. "We learned that the flight could be longer than expected," says Piccard, and "that we cannot just take off with the absolute certainty that the next five days will be OK." Avoiding bad weather systems means more unplanned diversions. "When we made simulations from Hawaii to Miami, we had to land in Phoenix, Arizona, because there was a big thunderstorm on the Gulf of Mexico," says Piccard. "So we learned to be more flexible."

Fair-weather flyer

Earlier solar planes also faced turbulent weather — with mixed results. *Gossamer Penguin*'s successor, *Solar Challenger*, completed its flight across the English Channel in 1981 on a sunny day with white puffy clouds. But Bob Curtin, who has worked at AeroVironment since the 1980s, recalls that "it was fairly turbulent actually, there were lots of clouds in the sky". However, *Solar Challenger* handled more like a small, light aircraft compared with the giant *Solar Impulse*.

The ultralight unmanned Helios craft, built by AeroVironment and NASA, didn't handle turbulence so well on its final flight. Its huge 'flying wing' structure was designed to flex into a curved shape when flying, and was able to handle moderate turbulence. As part of NASA's mission to build high-altitude and long-endurance craft it flew at altitudes above 29 kilometres. But during an attempt to set a longer flight record in 2003, the curved wing started oscillating uncontrollably, and the structure broke up over the Pacific Ocean. Unlike Helios, *Solar Impulse*'s design follows a classic rigid-wing structure, so rather than oscillate, the craft will get knocked around by the wind. Piccard says that the main reason for choosing this design, however, was the need to incorporate the cockpit.

Given the unpredictable nature of the weather, the multinational team building *Solar Impulse* is perhaps wisely sticking to known technologies for the final design. A feasibility study done in 2003 predicted the technology

André Borschberg (left) and Bertrand Piccard will pilot *Solar Impulse* on its round-the-world attempt.

SOLAR IMPULSE/STÉPHANE GROS

Solar aviation highs and lows

The first recorded solar-powered flight was on 4 November 1974, when Robert Boucher of Astro Flight launched his remotely controlled Sunrise I by catapult in the Mojave Desert.

The first manned solar aircraft was *Gossamer Penguin* (pictured below, left) — a smaller version of the human-powered *Gossamer Albatross*, which crossed the English Channel in 1979. Designed by Paul MacCready of AeroVironment in Monrovia, California, *Gossamer Penguin* flew a distance of more than 3 kilometres and had a wingspan of 22 metres. Its solar panels provided just 541 watts of power.

Gossamer Penguin's successor, *Solar Challenger* (pictured below, centre), crossed the English Channel, covering 262 kilometres, in 1981. Despite its small wingspan of just 14 metres, its solar cells provided about 2,600 watts and the craft reached an altitude of 4.4 kilometres.

AeroVironment went on to collaborate with NASA to design unmanned solar planes for satellite replacements and reconnaissance missions. Together, they launched Helios (pictured below, right), an ultralight 'flying wing' with 14 motors and a 75-metre wingspan. Helios's prototype reached an official world-record altitude for non-rocket powered aircraft of 29.5 kilometres in 2001 and sustained flight above 29 kilometres for more than 40 minutes. Helios broke up over the Pacific Ocean in 2003.

Civilian exploits include *Sunseeker*, a manned combination of a glider and solar-powered plane, in which Eric Raymond crossed the United States in 21 stages over almost two months in 1990. In 2005, Alan Cocconi's remote-controlled solar plane, SoLong, stayed aloft for 48 hours. Raymond is part of the team working on *Solar Impulse*, and Cocconi is a key adviser.

Cocconi's record for the longest unmanned flight was broken in 2007 by Zephyr, a plane from the British defence company QinetiQ, which flew for 54 hours. **V.C.**

B. RHINE/NASA

J. READER/TIME LIFE PICTURES/GETTY IMAGES; N. GALANTE/PMRF/NASA

improvements that were likely to be available in 3–4 years time, but didn't plan on any technological breakthroughs.

For instance, the study predicted that monocrystalline solar cells would have efficiencies of 20% — they now provide around 22%. At just 130 micrometres thick, the solar cells are flexible enough that they can be integrated into the upper surface of the wings without shattering. The team also correctly predicted that the energy storage density of rechargeable lithium batteries would reach 200 watt hours per kilogram.

The design is now frozen with these technologies, so the challenge is one of engineering rather than science. "The technology is given, so you have to reduce energy consumption," says Borschberg.

Testing times

Now that the project has three of four major sponsors in place, and two-thirds of the funding it needs, the team has started to build a smaller prototype. Test flights with the 61-metre prototype, scheduled for later this year, should give a better idea of how feasible overnight flights are. Once the lessons learned from the prototype have been fed back into the overall design, the team plans to build the full-size plane during 2009–10, with the round-the-world mission slated for 2011 if all goes well.

When *Solar Impulse* finally gets airborne, its progress will be watched carefully. "We're very interested in *Solar Impulse*," says Kelleher, who, as a stunt pilot himself, would love a chance to fly the plane. In its quest for energy efficiency and low weight, he sees *Solar Impulse* as "the art of the possible". But his company is more interested in unmanned solar planes as an alternative to satellite technology for the communications industry. Because of the cost of transmitting data from Earth to satellites, for example, Zephyr could provide a cheaper way to relay information. "We don't expect it to replace satellites, but it may be able to do many of the jobs that satellites can't do so well, or do them much more cost effectively," Kelleher says.

> "The real future is probably in an unmanned vehicle."
> — Bob Curtin

Curtin, now vice-president of business development at AeroVironment, also sees a future for solar-powered planes, although the company's solar research has been on hold since the Helios crash. He says that everything the firm learned about low-powered flight is being applied to its Global Observer project, an unmanned high-altitude aircraft for communications relay and observation. Global Observer will be fuelled by liquid hydrogen in an internal combustion engine, and will fly for a week at a time.

The reason the company decided to go for hydrogen and not solar on this project, says Curtin, is that the payload of the Global Observer is large — around 181 kilograms — much bigger than the 30-kilogram Zephyr. Another limitation of solar-powered flight is that current technology requires long days and short nights — restricting the range of the craft to lower latitudes.

But Curtin doesn't rule out a return to solar power as solar cells and battery technologies improve. "The real future is probably in an unmanned vehicle," he says, "because you're trying to make something fly perpetually, and if you do that, it doesn't make sense to have a human on board."

Piccard is optimistic that his dreams of fuel-free flight, like MacCready's before him, will inspire others to change their thinking about energy consumption. When asked why they pursue such missions, both Piccard and MacCready cited the example of Charles Lindbergh's solo Atlantic crossing in 1927. "Lindbergh was alone because the rest of the payload had to be gasoline," notes Piccard, yet 35 years later aircraft crossing the Atlantic were able to carry 300 passengers. This solo pilot is gambling he won't be alone forever. ■

Vicki Cleave is a senior editor for *Nature Materials*.

Non-traditional publishing choices can enrich science

SIR — The paramount importance of publishing in biology dissuades many young scientists from making non-traditional choices with regard to where and how we publish our work. My colleagues and I believe it is in our own interests to identify the shortcomings of traditional publishing and to explore other publishing possibilities that are free of those problems.

What can we do? First, learn about our options. There are several innovative developments poised to change the publishing landscape dramatically. Video publications, preprint archives and high-throughput online journals are but a few that have recently surfaced (for a discussion, see www.harvardpublishingforum.com). The onus is on all of us to investigate these resources and to consider how they might enrich our science.

To make a difference, we also need to contribute. Frustrated by technical difficulties in reproducing published experiments? Then publish a video protocol in the *Journal of Visualized Experiments*. Have you benefited from a colleague's comments at a conference? Then extend the experience, and comment on articles published by *PLoS One* and posted on *Nature Precedings*. These initiatives will take hold and achieve their full potential only with strong support from the scientific community.

If we collectively embrace these ideas, publishing will become more effective. Although the psychological and social barriers to submitting a contribution initially are surprisingly high, becoming involved has proved to be rewarding. Ultimately, scientific progress and the published record have a symbiotic relationship — improved communication will enhance the pace, progress and efficiency of research.

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Pakistan needs a powerful ethics and integrity body

SIR — Your Editorial 'The paradox of Pakistan' (*Nature* **450,** 585; 2007) highlights the importance of continuing reforms to the country's science and higher education. It is also crucial to establish an independent and powerful statutory body that oversees scientific research in Pakistan, to ensure that it complies with the universal norms of research ethics and integrity.

This body would make sure that all educational institutions throughout the country have such programmes in place, while recognizing that the three major elements of research ethics — individuals' autonomy, beneficence and justice in human-subject research — also conform with Islamic values. Unification of the Muslim world's intellectual resources with those of the rest of the world will help accelerate the pace of scientific discovery.

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Albedo-watching satellite needed to monitor change

SIR — Stewart Brand's Essay 'Whole Earth comes into focus' (*Nature* **450,** 797; 2007) makes a strong case for continuous satellite observations of the "whole Earth". A key reason is that existing observations are inadequate to monitor changes in global albedo — the amount of sunlight reflected by Earth and a key determinant of Earth's climate.

A comparison of existing albedo measurement programmes, which are based on polar-orbiting and geostationary satellites, shows large discrepancies in trends taken over several years, as well as poor correlations in monthly anomalies (N. G. Loeb *et al. J. Clim.* **20,** 575–591; 2007). In addition, there is a significant difference between the historical reflected flux data of the Earth Radiation Budget Experiment and the Clouds and the Earth's Radiant Energy System (CERES), and a large imbalance in the amount of incoming and outgoing radiation derived from the CERES measurements (F. A. Bender *et al. Tellus* **58A,** 320; 2006). The causes of these discrepancies are unknown and call for independent high-quality data.

DSCOVR, the radiometric satellite that Brand mentions — which is "mothballed" but ready to launch — would provide the data needed. From its position 1.5 million kilometres away at the Lagrange-1 point, it would orbit the Sun in synchrony with Earth and provide a continuous, well-calibrated proxy for global albedo by observation of the sunlit side of Earth. Understanding this albedo proxy could be helped by simultaneous diagnostic observations from the CALIPSO satellite (which measures the reflected laser light) and from the suite of instruments comprising the A-train satellite constellation, which includes CERES. It is therefore a matter of urgency to launch DSCOVR soon, in order to achieve synergy with existing satellites and to provide a bridging link with future systems.

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Citations in supplementary information are invisible

SIR — I would like to draw attention to a substantial drawback in publishing supporting scientific data online, in supplements to the printed research paper, usually because of space limitations. Unfortunately, the additional citations in this supplementary information are invisible to those services that rely on citations as a measure of the 'quality' of journals or of individual scientists, using them to determine impact factor, h-index or Scimago journal ranking, for example.

This becomes obvious when looking under the article heading for any citation that is referenced only in the supplement, using search engines such as PubMed, Scopus, Web of Science or Google Scholar. None will indicate that the particular reference is cited in the paper's supplement. This omission will affect ranking calculations, particularly for journals that post details of experimental methods in their supplements.

Like it or not, ranking of scientific achievement by citation-based methods is an important part of the scientific system, and journals should make all their citations accessible to those who need accurate numbers. The solution to this problem seems quite simple: the citations in the supplement have to be incorporated into the reference section of the main text by the authors.

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References in *Nature*'s Extended Methods sections, which are online-only but fully integrated into the full-text and PDF, are indexed in external databases such as PubMed. Supplementary Information for *Nature*, presented as a merged PDF online separate from the article PDF, does not usually contain references; see http://tinyurl.com/2of24c — Editor, *Nature*.

Contributions may be submitted to correspondence@nature.com. Please see Nature's Guide to Authors at http://tinyurl.com/373jsv.

BOOKS & ARTS

ALUMNAE ASSOC, ROYAL VICTORIA HOSPITAL TRAINING SCHOOL FOR NURSES

Architectural healing

A detailed history explores the symbiosis of modern scientific medicine and hospital design.

Medicine by Design: The Architect and the Modern Hospital, 1893–1943
by AnnMarie Adams
University of Minnesota Press: 2008.
240 pp. $82.50

D. Kirk Hamilton

The twentieth century saw the beginnings of modern scientific medicine and a shift in hospital architecture. Did good design catalyse medical progress during this period, or did medical practice dictate what was built?

AnnMarie Adams's careful and delightful book analyses hospital design between the late-Victorian scientific revolution of the 1890s and the Second World War. Adams, an architectural historian at McGill University in Montreal, uses the city's Royal Victoria Hospital (pictured) as her centre-piece. Around it she weaves carefully researched stories of other hospitals, architects, physicians and governors involved in the development of North American hospitals.

Adams questions the assumption held by medical and architectural historians that hospitals passively reflect evolving practice and innovation in medicine. She explores how physicians and architects worked together to invent the modern hospital. As hospital architects learned more about medicine, they began to offer practical and innovative solutions, as in the case of lighting for operating theatres and choice of materials that support hygiene. At the same time, some physicians acted as consultants on multiple design projects and lectured at conventions on design topics.

Medicine by Design contends that inter-war hospital architecture "anticipated and produced medical practices that were broadly and socially conceived". Service to the poor at a time when few effective cures were available was replaced by a mission to treat the sick as medicine improved. Hospital buildings began to reflect social strata with separate facilities for the wealthy on upper floors. The evolving professional status of nurses brought nursing schools, and with it, highly regulated, supervised dormitory housing shaped by paternalistic concerns for young unmarried women leaving the protection of the traditional home.

Between the wars, hospital architects began to separate the functional planning of a building from its aesthetic design. Adams notes that hospitals were slow to adopt the unornamented style of modern architecture so popular in the 1920s and 1930s, favouring Georgian or neo-classical aesthetic. Well-ventilated pavilions, developed while medicine was dominated by the 'miasma' theory, gave way to block planning as Louis Pasteur's germ theory advanced. The latter provided more segregated spaces and greater distances from open windows, and allowed physicians to control airflow and cleanliness.

As surgeons made the transition from gentlemen's attire to gloves, masks and gowns, and used Joseph Lister's carbolic acid spray on wounds, architects designed new operating theatres with abundant natural and artificial light, space for new equipment, and resilient surfaces that could be cleaned with harsh chemicals. The prevailing 30-bed wards were replaced by separate rooms of two to eight beds each, arranged along both sides of a central corridor. These rooms and spaces for large machines such as Wilhelm Röntgen's X-ray apparatus or for elaborate hydrotherapy were better suited to buildings with a broader beam span than the typical pavilion.

Adams begins her story with the 1893 opening of the Royal Victoria Hospital, a tribute to Queen Victoria's golden jubilee in 1887 conceived by local civic leaders and physicians with Scottish links — at the time many North American physicians were trained in Edinburgh. British architect Henry Saxon Snell's hospital design drew on the style of a Scottish baronial manor.

Using a wide variety of original sources, Adams describes the evolution of the Royal Vic and other North American hospitals through a succession of projects by prominent and lesser known architects. The Royal Vic and the Johns Hopkins Hospital in Baltimore, Maryland, completed five years earlier, were the first in North America to reflect the new thinking associated with the scientific revolution while still adhering to pavilion-style plans. Physician John Shaw Billings designed Johns Hopkins in collaboration with architects John Niernsee and Cabot & Chandler after touring many of the best European hospitals. Pavilions had become the

prevailing design there following the competition to rebuild the Hôtel-Dieu in Paris after the disastrous fire of 1772. Florence Nightingale supported pavilions, advocating hospital wards with large windows between every two beds, but criticized the design of the Royal Vic, where she felt that nurses on the wards would struggle to observe their patients efficiently.

Adams explores the important contribution of Edward Stevens, arguably the first specialist architect of hospitals. Stevens trained at Massachusetts Institute of Technology and made rigorous studies of European and North American hospitals and the practice of medicine. He designed, with Frederick Lee, numerous hospitals across the United States and Canada and wrote the influential guide *The American Hospital of the Twentieth Century* in 1918. Also influential were physician consultants such as S. S. Goldwater, the superintendent of New York Hospital. Goldwater proposed a design in 1905 for an urban high-rise hospital exploiting the new technologies of structural steel, elevators and electric lights. Although never built, the proposal was quickly followed by numerous designs for such multi-storey hospitals.

With medicine progressing ever faster, Adams' history reminds us why hospital architects and physicians should work together to optimize healthcare environments. ■

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Quest for extraterrestrial life

The Living Cosmos: Our Search for Life in the Universe
by Chris Impey
Random House: 2007. 416 pp. $27.95

Bruce Jakosky

From only one example, that of life on Earth, we have learned a lot about what makes a planet habitable. We know how life functions, how it may have originated and some types of environment that support its existence. Yet we do not know how widespread life might be. To do so we must extend our knowledge past Earth, into the Solar System and beyond.

The possible existence of extraterrestrial life — be it in the form of microbes on Mars, biological forms on planets orbiting other stars, or, frankly, UFOs and aliens visiting Earth — is fascinating. Yet astrobiology is firmly science-based rather than speculative and aimed at a curious public. Its major questions can be answered empirically with appropriate spacecraft and telescopes. To elucidate the origins of life, we might ask for example, is Mars habitable? Can signs of life be found there or on moons such as Jupiter's Europa and Saturn's Titan? Are there Earth-like planets with environments conducive to life around other stars? Might there be civilizations elsewhere in our Galaxy? If so, could we listen in on their conversations?

These questions run through *The Living Cosmos*. Astronomer Chris Impey provides a broad, accessible context for his thoughtful, engaging and up-to-date take on the quest for extraterrestrial life. The start and the end of the book pose questions about the relationship between art and science, and the likelihood of extraterrestrials having either. The rest dwells mainly on the science. Starting with the historic foundations of the field during the copernican revolution and following the story through modern science's development, key chapters deal with the origins of life, life in extreme environments and evolution here on Earth. The story then moves out into the Solar System and planets around stars other than our Sun. A far-reaching final chapter tackles intelligent life, interstellar travel, and the meaning of the search for life. Impey details current and future space missions and extrasolar planet research.

How is this exploration progressing and what lies ahead? Mars is arguably the best place to look for present or past life, given the evidence for liquid water there and the planet's proximity to Earth. Although many spacecraft have been sent, the future of the programmes is uncertain. Five craft currently send back data: the exploratory robot rovers Opportunity and Spirit from its surface, and orbiters Mars Odyssey, Mars Reconnaissance and Mars Express. In the United States, the next missions are over budget or delayed, notably the Mars Science Laboratory (scheduled for a 2009 launch) and the Mars Scout orbiter (now scheduled to launch in 2013). Despite repeated calls for a Mars Sample Return, plans for that or other missions beyond 2013 are unclear, especially in light of the recently released proposed budget for next year.

Beyond Mars, several moons in the outer Solar System are worth exploring. Jupiter's satellites Europa, Ganymede and Callisto are good targets because they may have recently hosted liquid water. Saturn's moons Titan and Enceladus, targets of the ongoing Cassini mission, are of great interest because of the abundance of organic molecules (especially on Titan) and the potential for near-surface liquid water (Enceladus). NASA is studying possible missions to these objects as a prelude to a major push mission into the outer Solar System.

To explore these remote places and perform science there, we need new technologies. Returning a sample of martian rocks and soil involves lifting the matter off Mars' surface, sealed tightly enough to avoid releasing any captured organisms into the terrestrial environment, and then flying the capsule back to Earth. Spacecraft must survive harsh radiation fields around the giant planets and perform difficult manoeuvres deep within their gravitational wells to orbit or land on a satellite.

These problems are solvable. But in our tight-budgeted times, technology development is often of lower priority than maintaining ongoing missions and starting new ones. Ironically, having the new technology in hand at the start of mission development helps prevent budget overruns later on. It is shortsighted to skimp on innovation. We pay the costs later, possibly in axed missions.

Recently, insufficient funding has postponed key astrobiology missions such as Terrestrial Planet Finder and Mars Sample Return. NASA and the science community must work together to address cost overruns and optimize research within the funding available. Once we have a viable plan of action, we will be able to answer the questions that Impey has ably outlined, and continue the search for life elsewhere. ■

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NASA

The icy surface of Jupiter's moon Europa may recently have hosted water: could it support life?

EXHIBITION

Cultures in the capital

Jane Rees

Our skin senses the world and projects our persona. This month in Liverpool, UK, 17 international artists incorporate skin and tissue cultures — either metaphorical or real — into artworks that reflect on how technologies may influence our future. The exhibition is part of Liverpool's European Capital of Culture 2008 celebrations.

The show is not for the squeamish. Examples of xenotransplantation, stem-cell research and genetic modification are among the exhibits at *sk-interfaces* at the Foundation for Art and Creative Technology (FACT). Face-transplant surgeon Peter Butler of the Royal Free Hospital in London, who opened the exhibition, suggested that using the techniques of bioscience to create works of art allows us to "dream about possibilities".

TISSUE CULTURE & ART PROJECT/O. CATTS & I. ZURR

A tiny leather jacket grown in the lab from mouse cells.

One work asks whether we can grow leather without harming animals. In *Victimless Leather*, artists Oron Catts and Ionat Zurr have formed three tiny jackets by growing a mouse cell-culture over a polymer mould. The polymer biodegrades over time, leaving the minute skin garments. Is such technology 'victim'-free? Not necessarily — cell-culture medium that includes fetal bovine serum is required to keep the cells alive.

Other works are equally bold. Sitting gruesomely in jam jars are two artists' co-cultured epidermal cells, which have been grafted onto pig derma and then tattooed with images of endangered species. To fully appreciate this art, produced by Marion Laval-Jeantet and Benoit Mangin, a collector may choose to have it xenotransplanted onto his or her own skin.

The performance artist Orlan (famous for her 'Carnal Art' plastic surgery interventions in the 1990s) has patched together a Harlequin coat from a mixture of her own cells, animal cells and, most controversially, cells from a 12-week-old human fetus of African origin, purchased over the Internet. She asks us to contemplate our hybrid origins and even our concept of species.

The artist Stelarc has sewn an ear-shaped prosthesis onto his left forearm, constructed from silicon scaffold and stem cells; the surgery is shown on continuous video loop on a large screen. He hopes to have a microphone and Bluetooth transmitter installed so that his extra ear becomes an 'Internet organ' for the body.

Legislation affects art as much as science in Jun Takita's *Light Only Light*, a model of the artist's brain covered with 'transgenic' bioluminescent moss — alas, with no detectable glow. A notice states: "Due to uncertain legal status of displaying genetically modified plants, non-transgenic moss is shown here."

A brave exhibition, *sk-interfaces* should provoke reaction, thought and discussion. Conceding that some will find it "distasteful", north-west regional director of public health John Ashton, says: "It begins to raise the question about whether challenging art of this type has any limits." However, John Hunt, a clinical engineer from the University of Liverpool who helped to produce *Victimless Leather*, hopes the exhibition will "encourage people to think about the concepts and boundaries" of clinical techniques so they can personally decide whether there are ethical issues.

Sk-interfaces is the first of three exhibitions to be held at FACT as part of its Human Futures programme, celebrating Liverpool's stint as European Capital of Culture. Science events elsewhere in the city this year include the opening of the Victoria Gallery and Museum housing artefacts such as a prototype Geiger counter owned by James Chadwick, and clay sculptor Rod Harris's residency at the University of Liverpool for Exploring Creativity in Engineering and Science with Sculpture. ■

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***Sk-interfaces* runs at the Foundation for Art and Creative Technology in Liverpool until 30 March. See www.liverpool08.com for other European Capital of Culture events.**

Text book for space shakers

Gravitational Waves: Volume 1: Theory and Experiments
by Michele Maggiore
Oxford University Press: 2007. 572 pp. $90

Neil Turok

Gravitational waves are ripples in space-time that travel through space at the speed of light. They are emitted by astrophysical sources such as binary stars or colliding black holes, or by violent phenomena such as cosmic strings or inflation in the very early Universe. Because they affect matter only weakly, gravitational waves are very hard to detect. To do so we need large instruments — interferometers several kilometres on a side — with extremely sensitive quantum-limited detectors, only recently available. The very fact that gravity couples so weakly to matter means that gravitational waves travel almost unimpeded through any material between us and the source of the gravitational waves. So they can provide a unique, direct view of some of the most dramatic cosmic events, including black-hole collisions or even the Big Bang itself.

At least that's the theory. Gravitational waves are confidently predicted on the basis of general relativity, but we've not been able to directly measure them so far. Their detection will be a scientific triumph, opening a brand new window onto the Universe. Over the past three decades, the technology of gravitational-wave detection has been steadily advancing, and sophisticated instruments are now operating in Italy, Japan, Germany and the United States. Versions coming online within a few years should reach the sensitivities required to see the gravitational waves expected from distant collisions involving black holes or neutron stars. Far more ambitious space-based experiments such as the Laser Interferometric Space Antenna (LISA) — a mission jointly led by the European Space Agency and NASA — are also planned for launch within the next decade. A growing community of experimentalists and theorists is forming, focused on the huge challenges of instrumental design and data analysis.

Students and experienced researchers will welcome Michele Maggiore's timely and authoritative new text book of classic results, detailed applications to specific gravitational wave sources, advanced mathematical methods and experimental issues. The first of two planned volumes, *Gravitational Waves* provides a thorough grounding in the theory of gravitational wave emission and a description of the practical techniques involved. It gathers many scattered results from a wide literature into a detailed, coherent, pedagogical review. The second volume, still in preparation, will cover

EXHIBITION

Time revisited

Joanne Baker

Tatsuo Miyajima, artist and vice-president of Tohoku University of Art & Design in Yamagata, Japan, uses installations of electronic counters to explore time, life and death. This month a solo exhibition celebrating 25 years of his work, entitled *Art in You*, opens at Art Tower Mito in Ibaraki, Japan. It includes the artist's first large-scale piece since he represented Japan in the 1999 Venice Biennale.

Miyajima is known for composing his works from myriad digital light-emitting diode (LED) counters. Each one displays the number sequence 1 to 9 on a continuous loop, followed by a pause for zero. The counters are arranged in groups, either fixed or in motion. In past shows they have been sent whizzing around the gallery floor on robotic platforms, set drifting relative to one another in arcs on a wall or floated in a pool of water. The LEDs glow in various colours and the devices count at different speeds. Sometimes the changing numbers are simply projected through gallery spaces so that a viewer can watch them pass fleetingly across their body or try to catch them as if they were falling leaves.

Not displaying zero is a deliberate act. "Zero represents death," says Miyajima, explaining that it originally meant both 'emptiness' and 'infinity' in fifth-century India and that the latter definition was lost as the idea travelled to Europe. For him, the number cycles represent life and death, which in Buddhist thought are a continuous cycle rather than discrete events. He likens the counters to human experience: individuality is echoed in their speeds and colours, and their illumination is symbolic of life.

Part of the beauty of Miyajima's kinetic sculptures is in their choreography. Crowds of jostling counters create changing patterns of light. Miyajima's works ask the viewer to watch and reflect on the passing of time; their complexity nods to the mathematics of chaos, relativity, atomic physics, cosmological structure and ecology. He has exhibited worldwide, including at the Royal Observatory in Greenwich, London, and his works are held by galleries such as Chicago's Museum of Contemporary Art and the Art Gallery of New South Wales, Australia.

Some compositions are inspired by the environment. A set of new photographs that appear in this show came out of a series of workshops the artist held in Hiroshima and an island he visited in his youth. Miyajima also organized 'Artists Summits' on climate change in Kyoto in 2005 and 2007. He feels strongly that artists should use their creative skills for crystallizing and communicating ideas to serve the public. "Imagination is the power to think about others and to understand others' pain. It is essential to secure the world."

Art in You features HOTO (pictured), a 6-metre tower of 3,827 multicoloured digital LEDs. The name is derived from a Buddhist story of a jewelled giant tower that symbolizes the wonder of life. "I want to tell children that it is a miracle simply to be born," says Miyajima, who conceived the idea to express hope after the terrorist attacks of 11 September 2001. ■

Joanne Baker is *Nature*'s Books & Arts editor.

***Art in You* runs from 16 February until 11 May at Art Tower Mito, Ibaraki, Japan (http://artinyou.jp).**

COURTESY OF ART TOWER MITO/SHIRAISHI CONTEMPORARY ART & LISSON GALLERY

the astrophysical and cosmological sources of gravitational waves, and what we can hope to learn about them.

The book is accessible to students who have taken a course in general relativity; it takes off from standard graduate-level textbooks, deriving the main results. In some places, though, the language is imprecise or lacks clarity; in others, too much space is devoted to the details of very specific applications. Also, an unfortunate number of minor grammatical errors and typos are scattered through the text. Hopefully, this will not distract readers from the large body of excellent and useful material that Maggiore has assembled on an important new frontier of astronomy and of fundamental physics. I look forward to *Volume 2*, and even more so to the dawn of gravitational-wave astronomy. ■

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NEWS & VIEWS

COMPLEX SYSTEMS

Ecology for bankers

Robert M. May, Simon A. Levin and George Sugihara

There is common ground in analysing financial systems and ecosystems, especially in the need to identify conditions that dispose a system to be knocked from seeming stability into another, less happy state.

'Tipping points', 'thresholds and breakpoints', 'regime shifts' — all are terms that describe the flip of a complex dynamical system from one state to another. For banking and other financial institutions, the Wall Street Crash of 1929 and the Great Depression epitomize such an event. These days, the increasingly complicated and globally interlinked financial markets are no less immune to such system-wide (systemic) threats. Who knows, for instance, how the present concern over sub-prime loans will pan out?

Well before this recent crisis emerged, the US National Academies/National Research Council and the Federal Reserve Bank of New York collaborated[1] on an initiative to "stimulate fresh thinking on systemic risk". The main event was a high-level conference held in May 2006, which brought together experts from various backgrounds to explore parallels between systemic risk in the financial sector and in selected domains in engineering, ecology and other fields of science. The resulting report[1] was published late last year and makes stimulating reading.

Catastrophic changes in the overall state of a system can ultimately derive from how it is organized — from feedback mechanisms within it, and from linkages that are latent and often unrecognized. The change may be initiated by some obvious external event, such as a war, but is more usually triggered by a seemingly minor happenstance or even an unsubstantial rumour. Once set in motion, however, such changes can become explosive and afterwards will typically exhibit some form of hysteresis, such that recovery is much slower than the collapse. In extreme cases, the changes may be irreversible.

As the report[1] emphasizes, the potential for such large-scale catastrophic failures is widely applicable: for global climate change, as the greenhouse blanket thickens; for 'ecosystem services', as species are removed; for fisheries, as stocks are overexploited; and for electrical grids or the Internet, as increasing demands are placed on both. With its eye ultimately on the banking system, the report concentrates on the possibility of finding common principles and lessons learned within this medley of interests. For instance, to what extent can mechanisms that enhance stability against inevitable minor fluctuations, in inflation, interest rates or share price for example, in other contexts perversely predispose towards full-scale collapse?

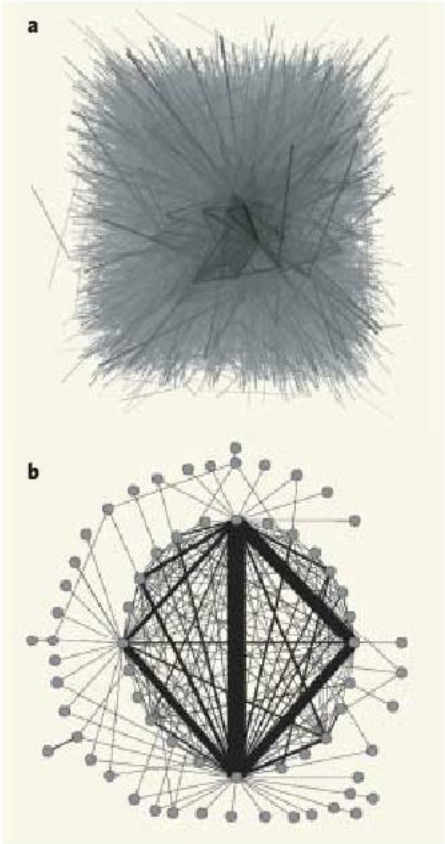

Figure 1 | The Fedwire interbank payment network. a, This 'furball' depiction takes in thousands of banks and tens of thousands of links representing US$1.2 trillion in daily transactions. **b**, The core of the network, with 66 banks accounting for 75% of the daily value of transfers, and with 25 of the banks being completely connected. Every participating bank, and every transaction, in the full network is known (akin to an ecologist knowing all species in an ecosystem, and all flows of energy and nutrients). So the behaviour of the system can be analysed in great detail, on different timescales and, for example, in response to events such as 9/11. (Reproduced from ref. 9.)

Two particularly illuminating questions about priorities in risk management emerge from the report. First, how much money is spent on studying systemic risk as compared with that spent on conventional risk management in individual firms? Second, how expensive is a systemic-risk event to a national or global economy (examples being the stock market crash of 1987, or the turmoil of 1998 associated with the Russian loan default, and the subsequent collapse of the hedge fund Long-Term Capital Management)? The answer to the first question is "comparatively very little"; to the second, "hugely expensive".

An analogous situation exists within fisheries management. For the past half-century, investments in fisheries science have focused on management on a species-by-species basis (analogous to single-firm risk analysis). Especially with collapses of some major fisheries, however, this approach is giving way to the view that such models may be fundamentally incomplete, and that the wider ecosystem and environmental context (by analogy, the full banking and market system) are required for informed decision-making. It is an example of a trend in many areas of applied science acknowledging the need for a larger-system perspective.

But to what extent can study of ecosystems inform the design of financial networks in, for instance, their robustness against perturbation? Ecosystems are robust by virtue of their continued existence. They have survived eons of change — continental drift, climate fluctuations, movement and evolution of constituent species — and show some remarkable constancies in structure that have apparently persisted for hundreds of millions of years: witness, for example, the constancy in predator–prey ratios in different situations[2]. Identifying structural attributes shared by these diverse systems that have survived rare systemic events, or have indeed been shaped by them, could provide clues about which characteristics of complex systems correlate with a high degree of robustness.

An example of this kind emerges from work on the network structure of communities of pollinators and the plants they pollinate[3]. These networks are disassortative, in the sense that highly connected 'large' nodes tend to have their connections disproportionately with 'small' nodes; conversely, small nodes connect with disproportionately few large ones.

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50 & 100 YEARS AGO

50 YEARS AGO

It was natural that the genetic transformation of bacteria effected by the introduction of foreign deoxyribonucleic acid should lead to speculation as to whether the phenomenon could also be induced in higher forms. That similar treatment should be capable not only of altering the racial characteristics of the growing vertebrate but that such changes would also be heritable seemed one of the least likely outcomes of such an experiment. Recently published reports by Benoit *et al.* state that they have succeeded in changing the characteristics of ducks of one breed by injections of deoxyribonucleic acid from another, and that the modifications continued to be identifiable in the progeny of the treated birds. Because of the importance that must be attached to such revolutionary claims, and in the absence, as yet, of substantive evidence from repeat experiments, the work of Benoit and his colleagues should be subjected to critical scrutiny.

From *Nature* 22 February 1958.

100 YEARS AGO

In the February issue of *British Birds* the editors discuss certain allegations against the black-headed gull which formed the subject of notice in the previous issue. Without entering into the controversy, we may notice that the allegations have induced two county councils in Scotland to strike gulls of all kinds out of the protected list. In another paragraph the editors refer to the subject of "luminous owls." In their opinion, the luminosity is most probably to be attributed to phosphorescent bacteria derived from decaying wood. It may, however, be due either to a phosphorescent feather-fungus (akin to one known to occur in geese) or to a diseased condition of the oil-gland, whereby the oil is more abundant than usual, and so abnormal in its nature as to become luminous on exposure to the air.

From *Nature* 20 February 1908.

The authors[3] show that such disassortative networks tend to confer a significant degree of stability against disturbance. More generally, ecologists and others have long suggested that modularity — the degree to which the nodes of a system can be decoupled into relatively discrete components — can promote robustness. Thus, a basic principle in the management of forest fires and epidemics is that if there is strong interconnection among all elements, a perturbation will encounter nothing to stop it from spreading. But once the system is appropriately compartmentalized — by firebreaks, or vaccination of 'superspreaders' — disturbance or risk is more easily countered.

As the report[1] notes, this is a complicated question, because modularity will often involve a trade-off between local and systemic risk. Moreover, the wrong compartmentalization in financial markets could preclude stabilizing feedbacks, such as mechanisms for maintaining liquidity of cash flows through the financial system, where fragmentation leading to illiquidity could actually increase systemic risk (as in the bank runs leading to the Great Depression). Redundancy of components and pathways, in which one can substitute for another, is also a key element in the robustness of complex systems, and effective redundancy is not independent of modularity.

In short, the dynamical implications of the topology of financial networks emerge as good candidates for further research. This is a lively field: the interplay between network topology and random or targeted 'attack' has also provided insights for the control of infectious diseases[4] and the defence of networks such as the Internet[5].

Following this theme, the Federal Reserve Bank of New York commissioned a study[6] of the topology of interbank payment flows within the US Fedwire service (Fig. 1); this is a real-time settlement system, operated by the Federal Reserve System, within which some 9,500 participating banks transfer funds. The sample from this network amounted to around 700,000 transfers, with just over 5,000 banks involved on an average day (ecologists studying food webs can only dream of such high-quality data). The authors[6] find the connectivity of this network — the ratio of the number of banks or nodes connected by one or more transfers to the total number of possible connections (essentially $0.5n^2$, where n is the number of banks) — is very low, around 0.003. This connectivity is characterized by a relatively small number of strong flows (many transfers) between nodes, with the vast majority of linkages being weak to zero (few to no flows). On a daily basis, 75% of the payment flows involve fewer than 0.1% of the nodes, and only 0.3% of the observed linkages between nodes (which are already extremely sparse). This kind of inequitability in linkage strengths (with most links being weak) is thought to predominate and help stabilize some ecological networks.

Overall, the topology of this Fedwire network is highly disassortative: large banks were disproportionately connected to small banks, and vice versa; the average bank was connected to 15 others, but this does not give an accurate idea of the reality in which most banks have only a few connections while a small number of 'hubs' have thousands. These strongly nonrandom and disassortative characteristics of the bank-transfer network are, as noted above, shared by some ecological systems. They also resonate with theoretical studies suggesting that sparseness of strong linkages can confer greater stability in systems whose components (nodes, banks, species) have some self-regulation[7,8].

These insights must be viewed against the reality that the payments system may not always be the relevant network for understanding systemic events. As the report notes, political and social networks may emerge to play a larger role in liquidity transactions and/or in the spread of rumours, which can ultimately influence the tides of fear and greed, and thence consensus valuation of markets. In this way the ever-changing finance problem, despite having certain resemblances to that posed in understanding ecosystems, is different from the fixed networks considered in physical sciences. The report puts it succinctly: "the odds on a 100-year storm do not change because people think that such a storm has become more likely". Emphasizing the point is this observation[1]:

"... in contrast to management of the electric power grid, there are only coarse or indirect options for control of the financial system. The tools available to policymakers — such as those used by central banks — are designed to modify individual incentives and individual behaviors in ways that will support the collective good. Such top-down efforts to influence individual behaviors can often be effective, but it is still difficult to control the spread of panic behavior or to manage financial crises in an optimal way. Within the financial system, robustness is something that emerges; it cannot be engineered."

Thus, although the study of payment flows is of immediate interest to central bankers, it may miss an essential aspect of systemic risk, namely the 'contagion dynamics' of public perceptions and asset valuation associated with the interaction of balance-sheets (the mutual financial obligations and exposures that link companies). For example, how contagious are inflated valuations of Internet stocks? Are there hidden, mutually dependent risks associated with such high valuations? It could be useful to examine the dynamic network of balance-sheets, and if possible to quantify the interactive effects of valuations, credit policies, hedging and so on among financial institutions, especially investment banks. Such balance-sheet networks could be helpful in studying the effects of asset-pricing bubbles, credit crises and the poorly understood but potentially worrying effects of the current widespread use of derivatives (futures and options) and dynamic hedging by investment banks to manage risk on the fly. Whatever the case, it seems that the ephemeral networks

that define financial reality and global markets are a key to understanding the ecology of market robustness and its potential vulnerability to collapse. ■

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MATERIALS SCIENCE

The gift of healing

Justin L. Mynar and Takuzo Aida

Synthesis of a rubber-like material that can be recycled might not seem exciting. But one that can also repeatedly repair itself at room temperature, without adhesives, really stretches the imagination.

When the Spanish conquistadores first witnessed the Aztec game played with a bouncing rubber ball, they thought that such balls must be possessed by evil spirits. Imagine their reaction if, on cutting the ball in half, it was made as good as new simply by pressing the two halves together without heat or adhesives.

Even today, such a feat would have a touch of magic about it. But this is what Cordier *et al.* (page 977 of this issue[1]) have achieved. They have synthesized a material with the properties of rubber that, when severed, can self-heal at room temperature if the severed surfaces are brought together. The underlying mechanism is that of 'supramolecular assembly', which operates through non-covalent interactions. The material maintains its mechanical features even when repeatedly broken and repaired.

Self-healing materials have long been the subject of research, with many groups exploring systems that undergo reversible transformation from individual components to a composite, or systems that incorporate self-healing materials within the composite. The potential applications are manifold — tears in clothes that effectively stitch themselves together; long-lasting coatings and paints for houses and cars; and, to take one example on the medical front, self-repairing artificial bone and cartilage.

One of the milestones in this field came from White *et al.*[2], whose approach involved mixing microcapsules containing healing monomers into an epoxy polymer matrix that contains a catalyst. When a break occurs, the microcapsules are destroyed, releasing the monomers into the crack; there they come into contact with the catalyst, triggering a polymerization reaction that swells the healing material until the crack is closed. This system needs no intervention when healing. But if a fracture occurs again in the same place, there is less or no healing agent to effect a repair. This problem is being addressed by the use of 'microvascularization' to resupply healing agents, but it is difficult to construct the necessary channels, and the approach is limited to small-scale operations[3].

Chen *et al.*[4] developed another strategy. They used polymers modified with moieties that undergo reversible cross-linking — meaning that varying decomposition and formation of the composite occurs, thus creating a self-healing system. An advantage of 'reversible systems' such as this is the ability to self-mend repeatedly, even after several fractures have occurred at the same place. But it is not easy to synthesize these polymers, and an external stimulus (in this case, heat) is required. The authors claimed that the system can repair itself under mild conditions, but the temperatures used were well above room temperature.

The beauty of Cordier and colleagues' work[1] is the clever use of supramolecular assembly to create a material that not only has the properties of rubber, but is also self-healing. Conventional rubbers are typically long-chain, cross-linked polymers that can stretch then recover to their original size and shape. Instead, Cordier *et al.* achieve those properties with a mixture of small ditopic molecules (those that can associate with two other molecules) and multitopic molecules (which can associate with more than two). The resulting supramolecular network exhibits partial cross-linking, through hydrogen bonds, which means that the material does not crystallize and is elastic. When it fractures, the active ends of the hydrogen-bond network are exposed because the strength of the self-assembly association is lower than that of covalent bonds. The broken ends can be thought of as living, and can be recombined by simply bringing them together (Fig. 1): microscopic association produces macroscopic healing.

The longer the time for which the broken ends are brought together, the more bridging associations form and the better the recovered extensibility. The elapsed time between severing and bringing the ends together also matters: the molecules at each end will start to couple with their neighbours, eventually deactivating the ends. But at room temperature it is a week before the ability to self-repair is lost. The only limitation of the material is that one must bring the two ends together and allow at least 15 minutes for self-repair.

It is satisfying to see the strategy of supramolecular assembly increasingly being turned to practical ends. Previous developments include that described by Meijer and colleagues[5], who have produced materials that take advantage of the fact that reversible, non-covalent forces are sensitive to many different environmental factors. Because these materials are comparatively easy to make and reprocess,

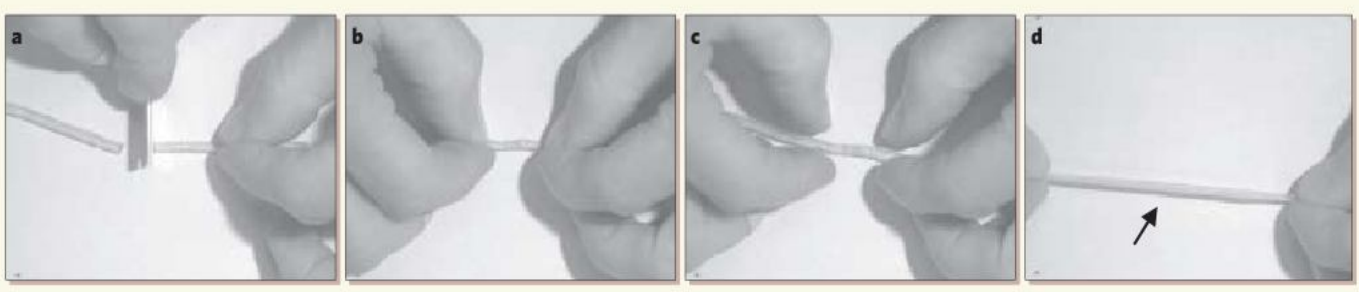

Figure 1 | On the mend. This sequence of photos, produced by Cordier *et al.*[1], shows self-healing being put to the test. **a**–**d**, Cut, join, mend, stretch.

they have attracted interest from industry. Indeed, one company, SupraPolix, produces supramolecular polymers with the ability to self-heal, although temperatures of 140 °C must be used.

Now, however, Cordier *et al.* have applied supramolecular chemistry to create a material that can heal at room temperature. Other chemists will soon be searching for, and designing and studying, a variety of small molecules to obtain specific mechanical properties in new self-healing materials. Cordier and colleagues' starting ingredients are fatty acids from vegetable oils, which offer a vast amount of variety for fine-tuning their material. Even now that material can withstand multiple fractures, needs no catalysts and is otherwise straightforward to produce (see Fig. 2 of the paper[1] on page 978). A final blessing is that it can be broken down with heat and easily recycled — so it is environmentally friendly, too. ■

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P.J. KEELING

Figure 1 | Evolutionary illumination in the round. These unassuming little brown balls, which are 5–7 micrometres in diameter, are cells of the marine alga newly described by Moore *et al.*[1]. In providing a connection between the apicomplexan parasites and their algal ancestors, the organism becomes a prime candidate for the complete genome analysis that should help lift more of the veil from ancient evolution.

EVOLUTIONARY BIOLOGY

Bridge over troublesome plastids

Patrick J. Keeling

Identification of a direct link between apicomplexan parasites and their algal ancestors is a development full of promise. It illuminates a dark corner in the evolution of photosynthesis, and further insights are to come.

One of the parallels between archaeology and evolutionary biology is that the most ordinary-looking objects can sometimes be the most revealing. An ancient ornament of unrivalled artistry might reveal little about the lives of the people who made it. But a monochrome pattern on a modest potsherd can, in the right context, furnish evidence that connects events contributing to an upheaval in human history.

On page 959 of this issue, Moore *et al.*[1] describe an analogous situation bearing on a problem in evolutionary biology. They describe a tiny marine alga that is superficially undistinguished: a 'little brown ball', like many other unexceptional marine microbes. But this little brown ball proves to be different, because it connects two pieces in one of the bigger puzzles of early evolution.

This puzzle is the interconnected histories of photosynthesis and of endosymbiosis, the process by which one organism engulfs another to mutual benefit, and which is a recurring theme in evolutionary history. The two pieces are apicomplexan parasites and dinoflagellate algae. Apicomplexa are exclusively intracellular parasites that cause many diseases, notably malaria. Therefore, it came as a surprise when they were discovered to contain plastids, the organelles derived from cyanobacteria that are responsible for photosynthesis in plants and algae[2,3]. The discovery of these 'apicoplasts' immediately sparked debate about their origins, because the closest relatives of apicomplexa, the dinoflagellates, also possess plastids. Dinoflagellate plastids are derived from the engulfment and retention of the plastid of a red alga. One side of the debate argues that apicoplasts evolved from this same red endosymbiont, which may have given rise to plastids in many other lineages as well[4–6]. The other side contends that apicoplasts are derived from an independent event, perhaps involving a green algal endosymbiont[7,8].

To distinguish between such disparate possibilities, one would normally compare the apicomplexan and dinoflagellate plastids and their genomes for clues to how they are related. But these plastids have proved curiously difficult to compare. The apicoplast could be forgiven for being a bit odd — it is, after all, found in non-photosynthetic, intracellular parasites — and not surprisingly all of its genes relating to photosynthesis have been lost[3]. The dinoflagellate plastid has no such excuses, yet it is arguably even stranger than the apicoplast: dinoflagellate plastid genes have undergone a mass migration to the nucleus, leaving only a handful of highly derived, mainly photosynthetic genes, and the genome itself has broken down into many miniature single-gene chromosomes[9].

The dilemma in comparing apicomplexan and dinoflagellate plastid genomes is therefore not simply that they are unusual, but that they are unusual in different ways. Most importantly, the absence in the apicoplast of genes that encode the photosynthetic machinery, and the corresponding removal of just about every other kind of gene from the dinoflagellate plastid genome, means there is virtually no overlap in plastid gene content: it is almost impossible to compare these genomes directly, and so their evolutionary relationship has remained stubbornly elusive.

This is where Moore and colleagues' alga comes in. While surveying coral symbionts, they found a little brown ball (Fig. 1). The sea is full of little brown and green balls, and they are often overlooked in favour of more interesting things. But further investigation of this particular little ball showed that it is special because, although it is a fully photosynthetic alga, it is also specifically related to the apicomplexan parasites. 'Missing links' do not really exist in the living world, but this is the next best thing because it has retained many ancestral features that were lost in apicomplexa and/or dinoflagellates.

Specifically, the new data provide a bridge between the non-overlapping gene content of the apicomplexan and dinoflagellate plastids, and so provide a definitive case that these plastids had a single endosymbiotic origin. Moore *et al.* show that housekeeping genes from the plastid of the new algal species are most closely related to apicomplexan homologues, while at the same time the photosynthetic proteins encoded in the plastid are most closely related to dinoflagellate homologues. At a stroke, this solves two outstanding puzzles: the apicoplast was derived from a red (as opposed to a green) alga in the same endosymbiotic event (as opposed to independent events) that gave rise to the dinoflagellate plastid.

This in itself is a fair day's work. But it is just the beginning. The unusual characteristics of apicomplexan and dinoflagellate plastids extend beyond their genomes to their membranes, pigments and protein-importing apparatus[10]. A similar situation is also true of their mitochondria, the organelles responsible for energy generation, where massive gene loss has accompanied the evolution of RNA editing and changes to the architecture of both the genome and the genes it contains[11]. In each case, further research on the new species could help reveal how such major transformations occur.

Indeed, its very existence also has implications for tackling much broader questions about how intracellular parasites evolved from algae, and whether the single origin of these plastids can be pushed even farther back in time to explain the origin of an even greater diversity of complex algae with red algal plastids, as proposed by the 'chromalveolate hypothesis'[5,6].

The discovery of an organism that falls at such an interesting junction in the tree of life happens only rarely, and this little brown ball will surely become the subject of a complete genome analysis soon after the ink of this commentary has dried. When it does, its genome will be a source of clues to many events that defined ancient evolutionary history. ■

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ORGANIC CHEMISTRY

Solid awakening

Leonard R. MacGillivray

Once dismissed as chemical graveyards, organic solids can in fact be manipulated to surprising effect: one example is a crystal designed to embark on a remarkable domino-rally of reactions when bathed in light.

A crystal, the chemistry Nobel laureate Leopold Ruzicka is reported to have said[1], is a chemical cemetery. Molecules in the solid state occupy the most restrictive of quarters: lying just ångstroms apart, the lively spontaneity of processes such as conformational change, enjoyed by molecules in solution, is largely forbidden to them. But although some effects of close packing might make molecules in solids relatively inert, this packing does determine important physical properties, such as a crystal's conductivity and reactivity. That fact has animated the field of crystal engineering[2], which in recent years has made great strides in controlling the properties of organic solids. As a case in point, Kuzmanich *et al.*[3], writing in the *Journal of the American Chemical Society*, describe how they have designed an organic solid that has truly remarkable 'chain reactivity'.

Reactions involving molecules are typically unimolecular (with just one player at the starting-gun) or bimolecular (with two). Both types of reaction occur less often in solids than in liquids, but they do occur. Kuzmanich and his colleagues focus on a unimolecular reaction: the photodecarbonylation of a ketone. A ketone is a molecule characterized by a carbonyl (C=O) group, the carbon atom of which forms a covalent bond to a carbon atom in each of two additional organic groups. When light excites such a molecule, the two carbon–carbon single bonds can break, generating reactive fragments known as radicals. If the conditions are right, the radicals combine, releasing the elements of the carbonyl group as carbon monoxide gas.

The breaking of the carbon–carbon bonds is reversible. In the solid state, this is an essential point: because the atoms of a crystal are locked in position by the lattice, the covalent bonds can easily re-form when the molecule returns to the ground state after the initial excitation[4]. Compared with the situation in the liquid phase, it is extremely difficult for the atoms to move and generate a newly coupled product with the release of carbon monoxide.

Kuzmanich *et al.*[3] set out to turn this wisdom on its head by designing a crystalline ketone that would ensure one bond breakage and in doing so make a second unavoidable. Their starting point is diphenylcyclopropenone, a ketone whose central carbonyl forms one corner of a triangular cyclopropene ring with three carbon atoms. The other two corners are connected to two identical phenyl (C_6H_5) rings (Fig. 1a–c).

The authors hypothesized that an initial bond breakage, caused by incident ultraviolet radiation, would release the large strain energy stored in the three-membered cyclopropene ring. Once unleashed, this energy would prevent the molecule from returning to the ground state, stopping the covalent bond from reforming. The energy released would at the same time promote the breaking of the second bond, casting the carbonyl group adrift as carbon monoxide. As a result, single crystals of diphenylcyclopropenone would react to produce solid diphenylacetylene (Fig. 1d).

This is wizardry, albeit of a more conventional chemical type. But a sign of something rather extraordinary was provided by the observation that the crystals took just minutes to crumble: a mark of a very efficient process. To quantify this observation, the authors determined the quantum yield of the reaction — the number of molecules that reacted per photon of incident light.

This is a notoriously difficult measurement owing to the differential scattering of light on

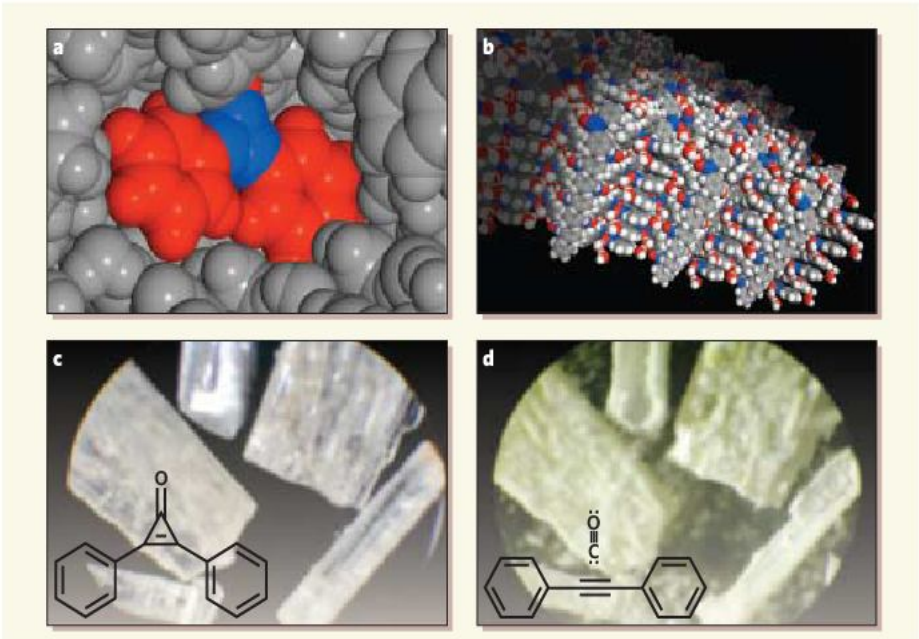

Figure 1 | Excited by light. **a**, **b**, These models, derived from X-ray data, show diphenylpropenone monohydrate in close-up (**a**; blue, cyclopropenone ring; red, phenyl and oxygen; remaining atoms, grey) and in their long-range structure (**b**; blue, cyclopropenone ring; grey, carbon; red, oxygen; white hydrogen). Nearest-neighbour diphenylpropenones are 4–5 ångstroms from each other. **c**, **d**, The before and after of Kuzmanich and colleagues' quantum chain reaction[1]: photoexcitation breaks a bond in the cyclopropenone ring (central triangle of structure), unleashing the strain energy pent up there. A second bond breaks, releasing carbon monoxide and leaving behind a powder of diphenylacetylene, $C_6H_5C\,CC_6H_5$.

different parts of a solid sample[5]. The authors turned to nanophysics for an answer to this problem[6,7]: specifically, the fact that a tiny crystal comparable in size to the wavelength of ultraviolet light (around 200–400 nanometres) can provide an environment homogeneous enough to minimize sample effects. Applying a technique developed by their own group[7], they used an aqueous nanocrystalline suspension to trap all the photons from the ultraviolet light source. Taking their earlier measurements on a related solid ketone as a reference, they were able to calculate an accurate value for the quantum yield of their new reaction.

That value was 3.3. For one photon to be activating more than one molecule (a quantum yield of 1.0), the reaction must be proceeding through a remarkable quantum chain process[8], with electronic excitations cycling through the crystal as bonds on different rings open and close. Any energy not used in the chain process probably led to loss of the included water (the crystal was prepared as an aqueous 'monohydrate'), the crumbling of the crystal, and recrystallization of the acetylene product.

In 1959, in his famous talk 'There's plenty of room at the bottom', Richard Feynman raised what was, in retrospect, an irresistible question[9]: "What would the properties of materials be if we could really arrange the atoms the way we want them?" Answers to this question can, and have, been sought in all states of matter — gas, liquid and solid. By effectively 'spring-loading' a molecule so that, when touched by light, it transferred its energy to a nearest neighbour, Kuzmanich *et al.*[3] establish a new connection between unimolecular and bimolecular reactivity. We can now start to wonder what further use we might make of the technique; whether, for example, the signal amplification provided by its domino-like behaviour might be useful for sensor-based materials and applications. With our rapidly growing knowledge of the structures and properties of organic solids[2], Ruzicka's morgue-like crystals will probably continue to reveal themselves as surprisingly lively places. ■

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EPIDEMIOLOGY

Emerging diseases go global

Mark E. J. Woolhouse

Novel human infections continue to appear all over the world, but the risk is higher in some regions than others. Identification of emerging-disease 'hotspots' will help target surveillance work.

The steady stream of outbreaks of new or unexpected infectious diseases is a much-discussed issue in the field of public health[1,2] and has even acquired its own dedicated scientific journal[3]. But for many years research has generally taken a case-by-case approach to understanding why new infections emerge. Now, Jones *et al.*[4] (page 990 of this issue) have published a systematic, quantitative analysis of recent global patterns of disease emergence. Their work provides insight into the ecology of emerging diseases, and has practical implications, providing pointers for the design of international surveillance programmes.

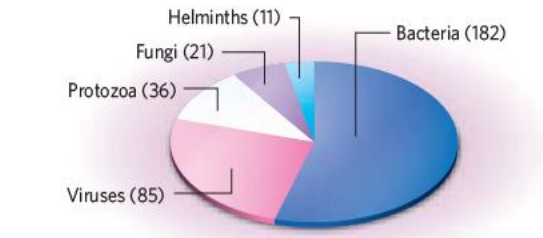
Jones and her colleagues began by collating data on what they call emerging infectious disease 'events' — that is, outbreaks of human disease associated with a new species or variant of an infectious agent (which could be any type of pathogen, from a virus to a parasitic worm). A painstaking review of the literature going back to 1940 turned up more than 300 such events, most of them involving bacteria (Box 1). (The database is published in full as supplementary information to the paper[4] and is itself a valuable resource.) The authors then quantified variation in the frequency of these events decade by decade across the world, and carried out a series of statistical analyses to look for relationships with other variables, ranging from human population growth to rainfall patterns.

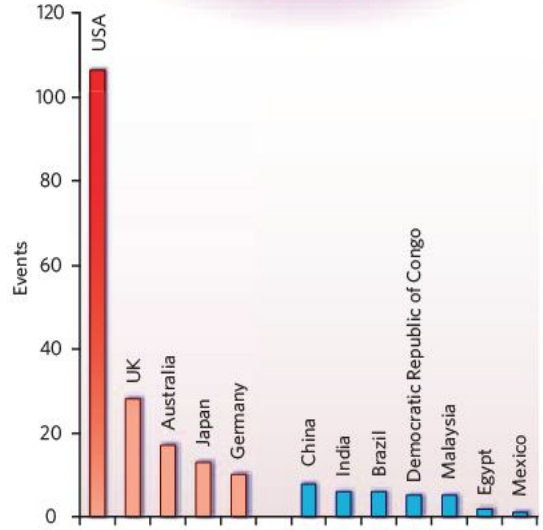
Box 1 | Emerging diseases: the pathogens and the places

In the work discussed here, Jones *et al.*[4] identified 335 emerging-disease 'events' reported worldwide between 1940 and 2004. The pathogens involved could be novel species or strains, including drug-resistant strains, of known species. Just over half of the events were associated with bacteria, as shown in the pie chart.

An example is *Escherichia coli* serotype O157:H7, first reported in the 1970s. This strain of the usually benign *E. coli* group is a food-borne pathogen that can cause fatal renal illness in the young and the elderly. It turned out to be just one type of verocytotoxigenic *E. coli* (VTEC): other VTECs have since been reported in the United States, the United Kingdom, Japan and other, mostly industrialized, countries.

In general, most reports of emerging-disease events come from developed countries; the bar chart shows the five countries with the most reports in red, with selected others in blue. In the United States alone, there have been more than 100 events reported (almost one-third of the total). Examples are infections with several species of hantavirus (such as Sin Nombre virus), fungal infections in hospital patients (including different species of *Candida*) and a range of bacterial infections acquired from animal reservoirs (for instance, *Bartonella henselae*, the cause of cat-scratch disease). Jones *et al.* suggest that this pattern reflects reporting bias. Often, the United States or another developed country can be merely the site of discovery of pathogens with wider distributions. This implies that there is still significant under-reporting of emerging infectious diseases from other regions of the world. **M.E.J.W.**





Before discussing their results, several issues that bedevil this kind of analysis should be acknowledged: how to define 'emerging'[5]; choosing the appropriate taxonomic unit of study[6]; making statistical allowance for groups of closely related pathogens sharing characteristics[7]; and ascertainment or reporting bias[8]. There are no definitive solutions to these problems, but Jones *et al.* fully explain and justify their approach, and are careful not to over-interpret their data.

The frequency of events rose to a peak in the 1980s and has since fallen (despite rising reporting effort). Jones and colleagues suggest that the peak might reflect the onset of the AIDS pandemic, creating a large (and still expanding) population that is highly susceptible to concomitant infections. The raw data also suggest that most events occur at higher latitudes — and particularly in Europe and North America (Box 1). This initially unexpected result is explained partly as an artefact of greater reporting effort, which, in turn, implies significant under-reporting in other parts of the world. Once reporting bias is accounted for, it becomes clear that, in general, most emerging infections are found where there are most people (rather than, as might have been supposed, on the remote fringes of human society).

Beyond these general patterns, there were some variations between different types of infection. Zoonoses (human infections shared with other vertebrates) were the most important category, accounting for 60% of events. This conclusion echoes that of earlier studies on the animal origins of human disease over both ecological and evolutionary timescales[9,10]. Jones *et al.* also confirm reports that zoonoses associated with wildlife were particularly important[11], but go on to show that the frequency of such events correlated with mammalian species richness (by contrast, no such correlation was found for infections associated with domestic animals). This result is neatly consistent with two previous observations: first, that many emerging pathogens have a broad host range[12]; and second, that a wide range of other mammals (and some birds) is associated with novel human pathogens[13].

Jones *et al.*[4] also stress the importance of drug-resistant infections. These account for more than 20% of events, mostly involving bacteria, and are especially common at higher latitudes — that is, in more developed regions where the use of antimicrobials is presumed to be greatest. Another major category is vector-borne infections. These also account for more than 20% of events and are currently on the increase, possibly linked to climate change, although other explanations cannot be ruled out.

Clearly, we must expect more infectious diseases to emerge in the near future. Jones and colleagues extrapolate their statistical analysis to generate risk maps that correct for current biases in reporting effort. The maps suggest that there are potential 'hotspots' of disease emergence — particularly in central America, tropical Africa and south Asia — that warrant greater surveillance. These findings support calls for international investment in the capacity to detect, identify and monitor infectious diseases, targeted at regions of the world where the need is greatest[14]. The benefits would not just be felt locally: in an era of increasing globalization, emerging infectious diseases are everybody's problem. ■

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GEOPHYSICS

Slab sliding away

Scott King

Does material that is subducted into Earth's interior at plate boundaries penetrate very far down? A model that links subsurface dynamics with the motion of the plates above provides a fresh approach to the question.

Much of the cold, brittle material at Earth's surface slides back down into the underlying mantle at subduction zones, where one tectonic plate dives beneath a second, overlying plate. By mapping the centres of energy of deep earthquakes[1], one can trace this subducting material as it moves down from the surface to depths of around 700 kilometres. But below this depth, earthquakes cease. Because of that, it was once assumed that subducted material descended no farther[2]. We now know this not to be the case, but what exactly does happen down there, and why, remains something of a mystery. On page 981 of this issue, Goes *et al.*[3] play their part in providing an answer, with a model that directly matches the fate of subducted material with the movements of plates at Earth's surface.

By virtue of the time it has spent at the surface, subducted material in Earth's interior is generally colder and denser than its surroundings, and can be imaged by seismic waves in a process similar to a medical ultrasound scan[4–6]. Thanks to this technique, we now know that, in some cases, subducted material extends well below 700 km. In other cases, it bends, buckles or flattens out horizontally at about this depth, making it difficult to trace further. Such deformation at 400–700 km is not surprising: this depth range corresponds to pressures at which mantle material undergoes several transformations between different solid phases[7], and at which its viscosity increases by between one and two orders of magnitude[8]. Both processes significantly alter the dynamics of deeply subducted material. But even armed with that knowledge, an explanation for the full range of behaviour seen in those geo-tomographic images has been elusive.

We still do not know, for example, what happens to the subducted material that flattens and deforms at about 700 km depth. Does it eventually sink to the base of the mantle (about 3,000 km below the surface), or does it remain trapped in the upper layer? The answer to this question would affect our ideas of transport between the upper and lower parts of the mantle, and thus would have profound implications for our understanding of the thermal and chemical evolution of Earth. Numerical models of mantle convection have shown that the sinking of cold material through the mantle varies in time[9,10]; we have an image of the mantle's structure only at the present day. So how can we look back at the mantle in past times?

Goes and colleagues' contribution[3] is to compare the record of plate motions with numerical models of subduction, and thus to identify tectonic events over the past 65 million years that indicate the descent of subducted material into the lower part of the mantle. The authors' model focuses on the first phase of subduction, when subducting material is driven downwards by its greater density ('negative buoyancy') alone, and sinks slowly into the mantle before it reaches the phase

transformations at 400–700 km down.

The authors identify four central points. First, subducted material sinks at a velocity that is controlled solely by its negative buoyancy and the viscosity of the surrounding mantle. Second, subducting material cannot bend in the time it takes to sink, owing to its internal strength. Third, there is a small 'subduction retreat' component that lessens the total sinking velocity: the subducting material sags under its own weight so that it rolls back somewhat as it subducts (Fig. 1). Fourth, because this subduction-retreat contribution is small, the velocity at which the subducting plate advances at the surface is close to the density-driven sinking velocity of the subducted material in the mantle.

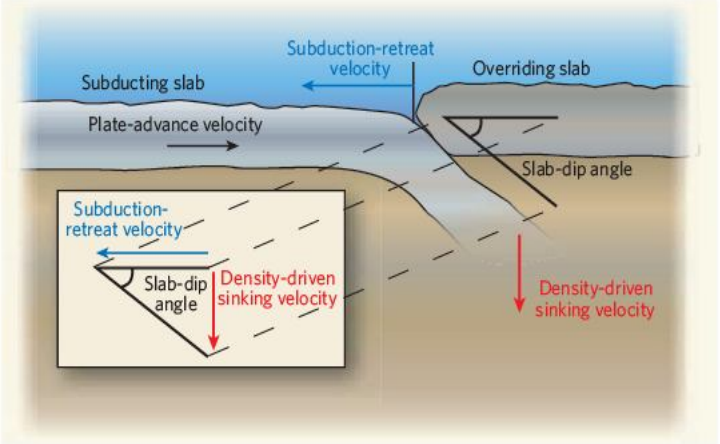

Figure 1 | Sink and retreat. In Goes and colleagues' free-subduction model[3], the velocity of a subducting slab, driven by its density, is intimately connected to the advance velocity of the plate on the surface. This implies that the subduction-retreat velocity, caused by the slab's own weight, is usually minor; the model neglects the forces caused by the overriding slab at the fault margin.

This 'free-subduction model' assumes that the contribution to the subduction-retreat velocity of the second, overlying tectonic plate is so small as to be negligible. If the plate-advance velocity is greater than the density-driven sinking velocity, the subducting material will thicken, increasing its negative buoyancy and thus its sinking velocity. It will do this until the sinking velocity is consistent with the plate-advance velocity. If, by contrast, the plate-advance velocity is less than the sinking velocity, the subducting material will thin out and stretch. Eventually, the bottommost part will drop off, forming a 'drip'.

For many subduction zones, the plate velocity is in good agreement with the sinking velocity predicted by this model. What is interesting is to look at the cases where the model fails. An example is the subduction zone that runs down the western coast of Central America. Here, an upper mantle of locally low viscosity, or a locally high density of the subducting material, must be invoked to match the model velocity to observations — although there is no evidence that the region has such exceptional properties. This is also a region where seismic tomographic imaging shows subducted material extending down beyond 700 km, and Goes *et al.* suggest that this extra material, not accounted for in their model, is the source of extra density driving the higher velocity. They go on to postulate that instances where the model fails are indicators of material sinking into the lower mantle, and they look for other times in the plate record where plate motions are outside the estimates for density-driven sinking velocities that they calculate.

The subducting material at the Central American subduction zone is relatively young. Young subducting material has had less time at the surface to cool, and its density contrast with the mantle material is relatively small. It should therefore penetrate into the lower mantle less readily than the older, denser material found at some other subduction zones — in contradiction to what is observed. Goes *et al.* point out a possible resolution: younger subducting material might be less dense, but it also drives less subduction retreat and has less intrinsic strength. It is therefore more readily deformed, making it more likely to thicken in that crucial transition zone 400–700 km down. Under those conditions, it will sink more readily into the lower mantle.

A caveat to Goes and colleagues' model[3] is provided by the simplification, mentioned earlier, that comes with ignoring the effect of the second, overlying plate on subduction retreat. Two locations serve to illustrate the difficulties such a simplification can bring. First, in the Mariana subduction zone in the western Pacific, old slabs seem to sink directly into the lower mantle. It is unclear whether this is because the slab material is dense enough to sink in spite of the phase transformations it undergoes, or whether it is the influence of other subduction zones in the region that exert local forces. Second, in the Hellenic Arc in the Mediterranean, the density-driven sinking velocity is almost exactly the subduction-retreat velocity: in other words, the plate-advance velocity is nearly zero. Clearly, there is a lot about subduction-zone dynamics that we still don't understand. But the work of Goes *et al.* gives us a framework in which we can begin to do that. ■

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CELL BIOLOGY

DNA versus membrane

Ling Juan Wu and Jeff Errington

Before it divides, a bacterium must move its replicated chromosomes away from the site of division, or risk having its DNA trapped in the membranes that separate the divided cells. How does it avoid this catastrophe?

In bacteria, chromosomal DNA spends most of its time floating in the cytoplasm without ever needing to venture across a membrane barrier. But when cells mate, or are about to divide, the DNA faces the challenge of traversing a membrane to reach its desired location. How does it meet this challenge? Writing in *Cell*, Burton and colleagues[1] find that, during spore formation in the bacterium *Bacillus subtilis*, the plasma membrane completely encloses the DNA in a process involving a channel-forming family of proteins called SpoIIIE/FtsK. These proteins are also known to be remarkable DNA-transfer engines that can pump DNA between cells.

When rod-shaped bacteria such as *B. subtilis* divide, their plasma membrane constricts in a ring. The ring then closes abruptly, forming two separate membranes (Fig. 1a). (To complete its division, the dividing bacterium must also synthesize new cell-wall material.)

Like most bacteria, *B. subtilis* has a single, circular chromosome. Chromosome segregation, which normally precedes membrane fusion, is a complex, multistep process in these organisms. It involves separating the replicated

circular sister chromosomes and moving them into the two resulting cells. If the replicated chromosomes fail to separate, strands of DNA could become trapped catastrophically in the closing division septum.

To deal with this problem, most bacteria use SpoIIIE/FtsK DNA transporters, which are located in the septum. Proteins of the SpoIIIE/FtsK family are found in many bacteria and consist of a transmembrane domain at one end (the amino terminus) connected by a linker of varying length to a DNA-translocating (ATPase) domain at the other end (the carboxy terminus). In *B. subtilis*, the SpoIIIE protein is particularly important because, when these cells form spores, they divide asymmetrically (Fig. 1a), and so the division septum always closes on a chromosome. Thus, for the smaller cell — the forespore — to have a complete chromosome, SpoIIIE must be able to transport about 3 million base pairs of DNA[2].

Burton *et al.*[1] address several aspects of the SpoIIIE-mediated transport process. It had been thought that a DNA-translocating motor complex forms from six SpoIIIE subunits, each being anchored to the leading edge of the septum through its hydrophobic transmembrane domain (Fig. 1b). The soluble ATPase domains of these subunits were thought to form an aqueous channel that prevented the two converging membranes from fusing, and that was large enough to allow the passage of only one of the two arms of double-stranded circular DNA to the forespore. Burton *et al.*[1] show that the two arms of the chromosomes are transported into the forespore simultaneously: therefore, at least two transporters would be required.

But the authors find that chromosome transport occurs after the septal membranes have fused. These results are surprising, as they imply a very different configuration of the SpoIIIE complex in which all the transmembrane domains of the SpoIIIE hexamer are embedded adjacent to each other in the same membrane lipid bilayer[1] (Fig. 1c).

Indeed, the authors isolate large complexes that could be two SpoIIIE hexamers from different bilayers linked through their extracellular loops. The transmembrane domains would then make up part of the transport channel. In other words, the transport channel consists of two linked SpoIIIE hexamers of opposite orientation, spanning both lipid bilayers and the cell wall sandwiched between them (Fig. 1c). Therefore, each chromosome arm crosses a series of distinct rings: the ATPase ring in the cytoplasm of the mother cell, the hydrophobic transmembrane ring, the extracellular (cell wall) ring and another round of hydrophobic and ATPase rings in the forespore.

To achieve unidirectional DNA transport through a channel with two motors in opposite orientations, one of the motors must be inactive. The SpoIIIE homologue in the bacterium *Escherichia coli* (FtsK) ensures unidirectional DNA movement by interacting with specific DNA sequences known as KOPS, which have highly skewed positions on the chromosome[3]. It is likely that *B. subtilis* SpoIIIE acts similarly, and that the polarity of the DNA molecule determines which motor is active. This is consistent with a report[4] that the position of the origin of replication on the DNA after septum formation determines whether DNA is transported into or out of the forespore. But these authors also believe that the SpoIIIE complex assembles only on the mother-cell side of the septal membrane, and that SpoIIIE works as a DNA exporter. Further analysis is therefore needed to reconcile this model with Burton and colleagues' data.

Previous studies have shown[5,6] that, during septum formation, SpoIIIE, which is initially distributed evenly throughout the membrane, condenses to a spot at the centre of the plane of division. It was thought that the protein then directly promotes membrane fusion to complete division. Moreover, SpoIIIE has been shown[6] to participate in another type of membrane-fusion event, called forespore engulfment, which occurs later in spore development. But Burton *et al.*[1] find that SpoIIIE is not needed for septal-membrane fusion, which is clearly at odds with these earlier proposals.

The assembly of SpoIIIE complexes probably occurs immediately before membrane fusion, with hexamers of the protein forming transport channels around each of the chromosome arms. Although Burton *et al.* find that membrane fusion occurs even in mutant cells lacking SpoIIIE, it is not clear exactly how under these circumstances the chromosomal DNA interacts with the lipid bilayers. Nor is it clear whether the DNA is in direct contact with the cell wall, and whether similar considerations hold in other bacteria. Furthermore, with the chromosome traversing the division septum, it would be interesting to know how the presence of two or more segments of double-stranded DNA affects septum closure by the division machinery.

One final puzzle posed by Burton and colleagues' work[1] is how the final loop of the circular bacterial chromosome passes through the two membranes. As the DNA does not seem to break during its transport, the two channels must either somehow open or fuse to allow completion of the transport process. Further biochemical, biophysical and cell-biological investigations should resolve these questions. ■

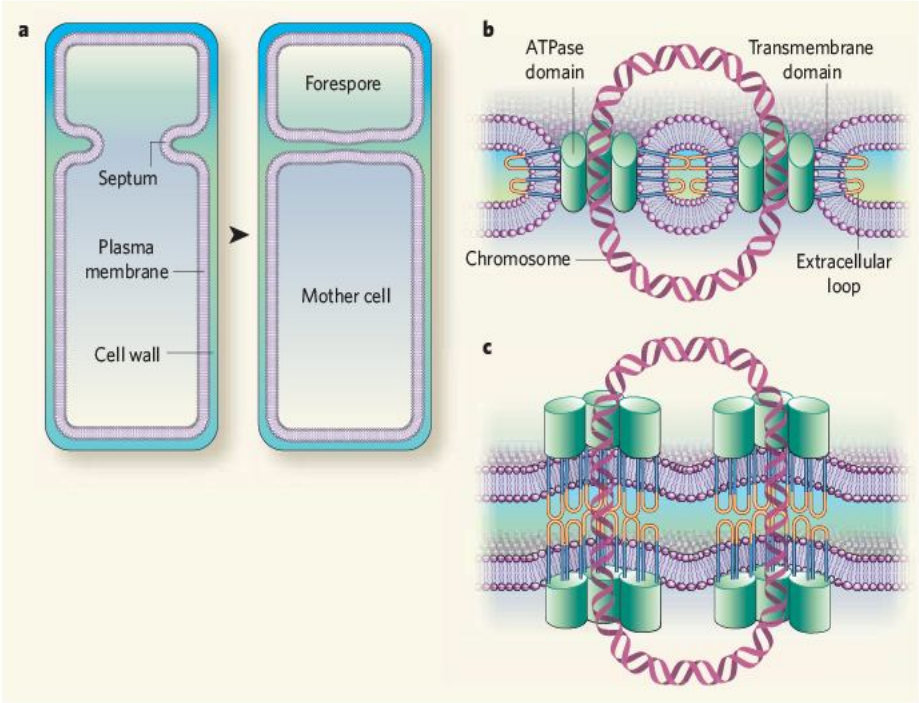

Figure 1 | Chromosome transport. a, When the bacterium *Bacillus subtilis* divides, its plasma membrane forms a constricting ring, which eventually fuses to form two separate membranes. The cell wall follows suit. Segregation of the replicated DNA is often incomplete when the plasma membrane fuses during normal growth, and is always incomplete in sporulating cells, which divide asymmetrically as shown. **b**, **c**, Two models of how proteins of the SpoIIIE/FtsK family might subsequently mediate translocation of the remaining DNA out of the septum. **b**, An earlier model suggests that SpoIIIE/FtsK proteins transport the DNA before the two leading edges of the plasma membrane fuse. **c**, Burton *et al.*[1], however, propose that the membranes fuse first, and that the DNA is then transported through two channels each consisting of six SpoIIIE subunits. In reality, the chromosomes are not as small or well defined as shown here, but extend well beyond the site of the pore.

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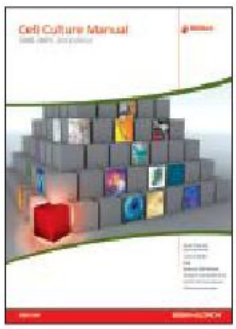

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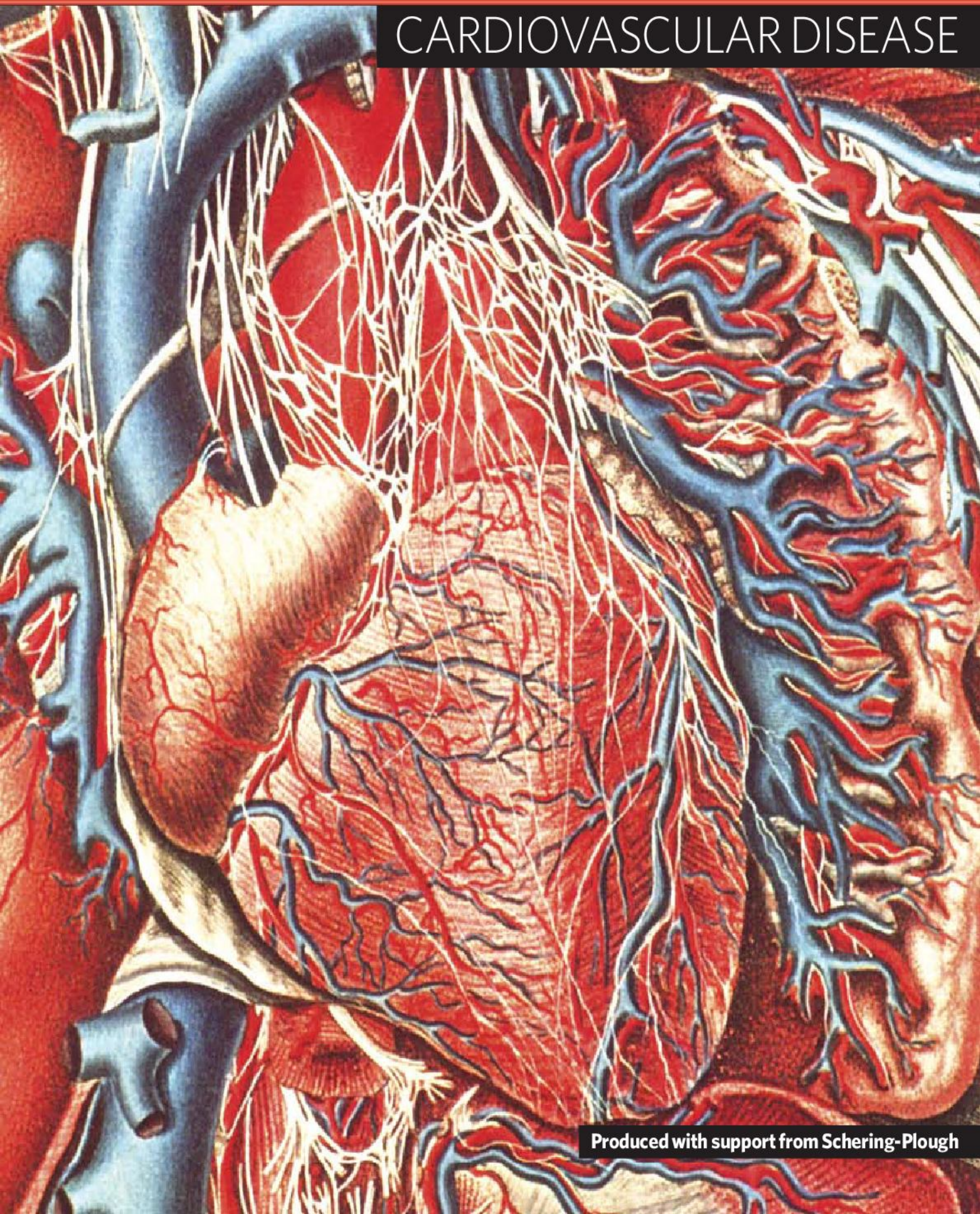

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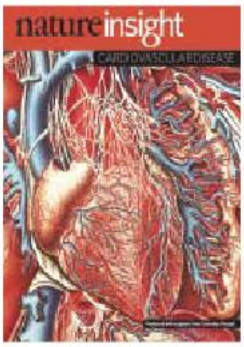

Cover illustration
The human heart, based on a drawing by Paolo Mascagni (1755–1815). (Courtesy of M. Kulyk/SPL)

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CARDIOVASCULAR DISEASE

Cardiovascular disease is the leading cause of death globally. According to the World Health Organization, it was responsible for 30% of all deaths in 2005. Although typically considered a disease of developed countries, its incidence is increasing in the developing world.

Cardiovascular disease usually stems from vascular dysfunction — for example, as a result of atherosclerosis, thrombosis or high blood pressure — which then compromises organ function. Most notably, the heart and brain can be affected, as occurs in myocardial infarction and stroke, respectively. For heart disease in particular, a wide range of underlying pathologies can lead to defective functioning of the heart muscle.

In the past few decades, major improvements have been made in treating some types of cardiovascular disease. In the case of coronary heart disease, for example, therapies such as the administration of statins and the insertion of stents have reduced death rates. However, new treatment options are urgently needed for all types of cardiovascular disease. Moreover, improving diagnosis is crucial, because by detecting the early stages of disease, the focus of therapy could be shifted from treatment to prevention.

This Insight brings together review articles about atherosclerosis, thrombosis, heart failure, cardiac arrhythmia and congenital heart disease. These articles explore recent progress in understanding the mechanisms that lead to disease and discuss the implications of these advances for identifying new therapeutic targets and developing new therapeutic strategies, including the potential use of stem cells for treating heart disease. Two progress articles also provide an update on how new technologies for identifying disease biomarkers and for imaging might enable disease to be detected at early stages.

We are pleased to acknowledge the financial support of Schering-Plough in producing this Insight. As always, *Nature* carries sole responsibility for all editorial content and peer review.

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Translating molecular discoveries into new therapies for atherosclerosis

Daniel J. Rader[1] & Alan Daugherty[2]

Atherosclerosis is characterized by the thickening of the arterial wall and is the primary cause of coronary artery disease and cerebrovascular disease, two of the most common causes of illness and death worldwide. Clinical trials have confirmed that certain lipoproteins and the renin–angiotensin–aldosterone system are important in the pathogenesis of atherosclerotic cardiovascular disease, and that interventions targeted towards these are beneficial. Furthermore, efforts to understand how risk factors such as high blood pressure, dysregulated blood lipids and diabetes contribute to atherosclerotic disease, as well as to understand the molecular pathogenesis of atherosclerotic plaques, are leading to new targets for therapy.

During atherosclerosis, the arterial wall gradually thickens to form an atherosclerotic plaque, resulting in the narrowing of the lumen of the artery. Consequently, the amount of blood supplied to the organ is reduced, most commonly affecting the heart and the brain. Plaques can abruptly rupture, causing a blood clot and often myocardial infarction (heart attack) or stroke. Intensive study of the cellular and molecular mechanisms that underlie atherogenesis (that is, the formation of atherosclerotic plaques) and plaque rupture has led to a consensus view of these processes[1] (Fig. 1). Initiation and progression of the lesion are highly complex processes, and many aspects of atherogenesis remain incompletely understood. Furthermore, in most cases, mechanistic insights have yet to be translated into therapeutic approaches. In this review, we discuss the most exciting advances in atherosclerosis research since 2000, emphasizing new findings that have translational and therapeutic implications. For a review of earlier findings, see ref. 2. At present, the two main conceptual approaches to therapy for atherosclerosis are manipulation of plasma lipoprotein metabolism or cellular cholesterol metabolism, and manipulation of inflammatory processes. Here we discuss both approaches, focusing on how recent findings might lead to new types of therapy. We set the scene with a discussion of how new therapeutic targets are identified and validated and then finish by looking at how genome-wide association studies are rapidly altering the way in which atherosclerosis is understood and might be treated.

Identification of therapeutic targets in humans and mice

Perhaps the most convincing evidence for a potential therapeutic target is provided when a human genetic condition arising from simple mendelian genetics is found to be associated with altered risk of atherosclerotic disease. An example is homozygous familial hypercholesterolaemia, which is caused by mutations in the gene encoding the low-density lipoprotein (LDL) receptor. The observation that this disease is associated with markedly premature atherosclerosis led to an understanding that increased concentrations of LDL cholesterol in plasma can cause atherosclerosis. This observation also led to the general concept that intervening to increase LDL-receptor expression would reduce LDL concentrations and thus the risk of atherosclerosis. However, classic mendelian disorders are not associated with most genes of interest, and even when they are, the prevalence of these disorders is usually too low to provide strong evidence of an association with atherosclerosis. By examining extended families, linkage studies have identified loci that seem to be important determinants of premature coronary artery disease, but it has often been challenging to identify the specific genes that cause disease. One notable recent success was the identification of a mutation in the gene encoding LDL-receptor-related protein 6 (LRP6) in a large family as responsible for autosomal dominant premature coronary artery disease accompanied by features of the metabolic syndrome (which is a group of risk factors that are commonly associated with coronary artery disease, including hyperlipidaemia, hypertension and insulin resistance)[3]. 'Candidate genes' are frequently tested by genotyping single-nucleotide polymorphisms (SNPs) in large cohorts (or groups) of patients and examining whether particular SNPs are associated with atherosclerotic disease. Unfortunately, many of the published association studies have not been subjected to rigorous replication[4]. Most recently, genome-wide association studies have been used in an attempt to identify genes that are significantly associated with atherosclerotic disease and its risk factors (discussed later).

Studies of genetically modified mice are also commonly used to identify and validate potential therapeutic targets, as well as to investigate atherosclerotic disease mechanisms in detail. The bidirectional flow of information between mouse and human studies has been crucial for furthering knowledge of atherosclerosis, as well as for validating new therapeutic targets. However, the relevance of mouse studies for understanding the pathophysiology of atherosclerosis in humans needs to be carefully considered. There are important differences between mice and humans with respect to two of the main processes involved in atherogenesis: lipoprotein metabolism and inflammatory pathways. In addition, there are many inconsistencies between the various studies of atherosclerosis in mice, and the basis of these discrepancies is often unclear. Strain differences might, in part, be responsible; indeed, there can be substantial genetic variation between control and experimental mice even after extensive backcrossing of both into the same strain. A lack of standardization in measuring lesion size in mice might also contribute to these discrepancies. Furthermore, there is an increasing recognition that lesion composition, rather than size, determines the acute complications of atherosclerotic disease in humans. However, compositional analysis of lesions in mice is not routine or standardized, and the implications of differing lesion composition for disease

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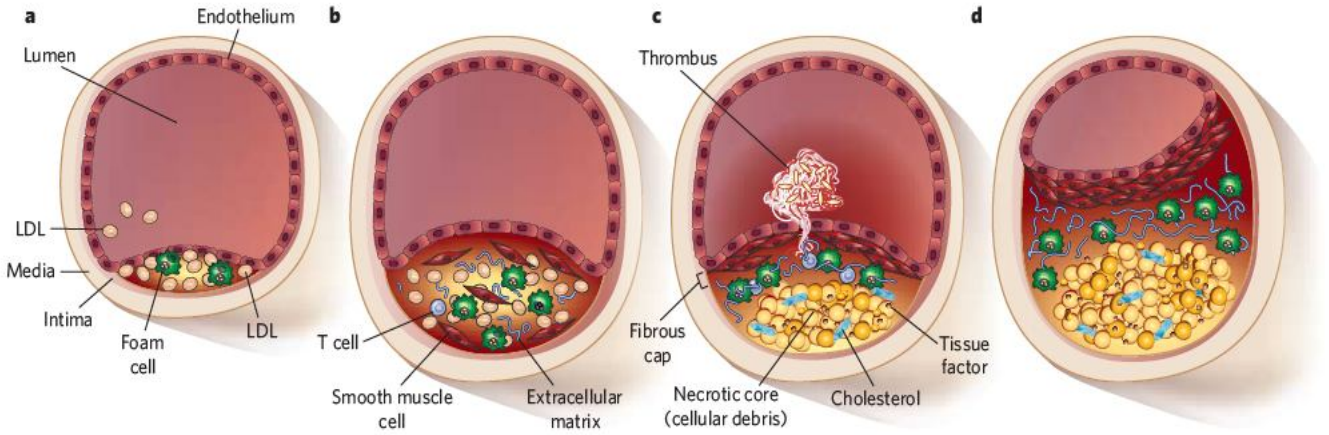

Figure 1 | Initiation and progression of atherosclerosis. Atherosclerosis occurs at sites in the arterial tree where laminar flow is disrupted. A lesion begins as a fatty streak (**a**) and can develop into an intermediate lesion (**b**), and then into a lesion that is vulnerable to rupture (**c**) and, finally, into an advanced obstructive lesion (**d**). A more detailed description of this process follows. **a**, Atherogenic lipoproteins such as low-density lipoproteins (LDLs) enter the intima, where they are modified by oxidation or enzymatic activity and aggregate within the extracellular intimal space, thereby increasing their phagocytosis by macrophages. Unregulated uptake of atherogenic lipoproteins by macrophages leads to the generation of foam cells, which are laden with lipid. The accumulation of foam cells leads to the formation of fatty streaks, which are often present in the aorta of children, the coronary arteries of adolescents, and other peripheral vessels of young adults. Although they cause no clinical pathology, fatty streaks are widely considered to be the initial lesion leading to the development of complex atherosclerotic lesions. **b**, Vascular smooth muscle cells — either recruited from the media into the intima or proliferating within the intima — contribute to this process by secreting large amounts of extracellular-matrix components, such as collagen. The presence of these increases the retention and aggregation of atherogenic lipoproteins. In addition to monocytes, other types of leukocyte, particularly T cells, are recruited to atherosclerotic lesions and help to perpetuate a state of chronic inflammation. As the plaque grows, compensatory remodelling takes place, such that the size of the lumen is preserved while its overall diameter increases. **c**, Foam cells eventually die, resulting in the release of cellular debris and crystalline cholesterol. In addition, smooth muscle cells form a fibrous cap beneath the endothelium, and this walls off the plaque from the blood. This process contributes to the formation of a necrotic core within the plaque and further promotes the recruitment of inflammatory cells. This non-obstructive plaque can rupture or the endothelium can erode, resulting in the exposure of thrombogenic material, including tissue factor, and the formation of a thrombus in the lumen. If the thrombus is large enough, it blocks the artery, which causes an acute coronary syndrome or myocardial infarction (heart attack). **d**, Ultimately, if the plaque does not rupture and the lesion continues to grow, the lesion can encroach on the lumen and result in clinically obstructive disease.

progression and outcome are not well understood. Another weakness of mouse studies is their focus on the mechanisms of lesion initiation and early progression instead of on the mature disease stages, which are the main stages targeted for therapy in humans. In addition, it is uncertain whether the vascular regions in which atherosclerosis is measured in mice have relevance to the human disease. These considerations translate into a lack of confidence that the effects of pharmacological interventions on atherosclerosis in mice will be reproducible in humans[5].

Lipoprotein metabolism

Lipoproteins transport lipids, including cholesterol, in the blood, and their metabolism is closely interrelated with the initiation and progression of atherosclerosis. The two most abundant lipoproteins in the plasma are LDLs and high-density lipoproteins (HDLs). Targeting aspects of their metabolism is one of the main interventions for preventing and treating atherosclerotic cardiovascular disease.

LDL metabolism

Both human studies and animal studies have shown that lipoproteins that contain apolipoprotein B (apoB) — for example, LDLs — are required for the development of atherosclerosis. The progression of atherosclerosis and the incidence of coronary and cerebrovascular events is significantly reduced, regardless of baseline LDL concentrations, after administration of inhibitors of HMG-CoA reductase (3-hydroxy-3-methylglutaryl coenzyme A reductase). These drugs, known as statins, inhibit cholesterol biosynthesis and result in accelerated clearance of plasma LDLs (the main lipid component of which is cholesterol) by the liver. The development of statins is one of the great translational successes in the atherosclerosis field and illustrates how research in biochemistry, cell biology, animal models, and human physiology and genetics can converge to produce a highly effective therapy.

The success of statins has inspired further efforts aimed at understanding the molecular mechanisms that regulate plasma LDL concentrations (Fig. 2). It has long been known that the amount of functional LDL receptor present at the hepatocyte surface is one of the most important factors influencing plasma LDL concentration. Recent research indicates, however, that LDL-receptor regulation is substantially more complex than previously thought. Genetic mutations that cause the rare disease autosomal recessive hypercholesterolaemia result in disruption of LDL-receptor recycling and in a substantial reduction in the number of LDL receptor molecules at the hepatocyte surface, thus markedly increasing plasma cholesterol concentrations[6]. The protein that is encoded by the mutated gene in individuals with this disease normally functions as a modular adaptor for the LDL receptor, either chaperoning the receptor to coated pits, where it binds to LDL, or anchoring the receptor in these pits during internalization.

More recently, another complexity was uncovered by genetic linkage analysis. Mutations in the gene encoding proprotein convertase subtilisin/kexin type 9 (PCSK9) are associated with a form of autosomal dominant hypercholesterolaemia[7]. An independent study found that mice on a high-cholesterol diet had a reduced expression of *Pcsk9* in the liver[8]. By contrast, hepatic overexpression of *Pcsk9* in mice results in marked hypercholesterolaemia[9], leading to the conclusion that the human mutations that cause hypercholesterolaemia have gain-of-function properties. Subsequently, PCSK9-deficient mice were found to have lower cholesterol concentrations than wild-type mice[10], and humans heterozygous for loss-of-function mutations in *PCSK9* were shown to have substantially reduced LDL concentrations[11], accompanied by a marked reduction in their lifetime risk for coronary artery disease[12]. Mechanistic studies show that after catalysing its own cleavage, PCSK9 is secreted and binds to cell-surface LDL receptors, thereby targeting them for degradation rather than recycling[13]. *PCSK9* is a sterol-responsive gene, the expression of which is upregulated by statin treatment, with the effect of blunting the reduction in LDL-cholesterol concentrations associated with statin therapy. PCSK9 is thus a highly attractive target for reducing LDL-cholesterol concentrations (Table 1).

Plasma concentrations of atherogenic lipoproteins such as LDLs are also influenced by the hepatic production rates of very-low-density

lipoproteins (VLDLs), the metabolic precursor of LDLs. Indeed, hepatic overproduction of VLDLs is a common finding in individuals with insulin resistance or type 2 diabetes and is also the basis of familial combined hyperlipidaemia, a common genetic lipoprotein disorder. Upstream transcription factor 1 (USF1) has been genetically associated with familial combined hyperlipidaemia[14], although the molecular mechanisms underlying the effect of USF1 on VLDL production are unclear. Studies of humans with low LDL concentrations have also provided important insights into the regulation of VLDL and LDL production. Mutations in the gene encoding apoB — the key structural protein component of VLDLs and LDLs —can result in low LDL concentrations, at least in part by reducing VLDL production. Patients with abetalipoproteinaemia have loss-of-function mutations in the gene encoding microsomal triglyceride transfer protein (MTP), which is required for loading triglycerides onto apoB. These mutations therefore result in markedly impaired VLDL assembly and secretion, and an absence of plasma LDLs. LDL concentrations in humans have been successfully lowered by inhibiting either the production of apoB-100 (the form of apoB that is produced by the liver and is present in LDLs) with antisense oligonucleotides or the activity of MTP with small molecules[15,16], and these strategies are in clinical development (Table 1). Genome-wide association studies are likely to identify other potential targets for decreasing LDL concentrations (discussed later).

HDL metabolism

As is the case for LDLs, the main lipid component of HDLs is cholesterol. However, in contrast to LDL cholesterol, plasma concentrations of HDL cholesterol are inversely associated with atherosclerotic disease. HDL metabolism is complex and is influenced by numerous factors (Fig. 2). HDLs have long been considered an important endogenous factor that protects against atherosclerosis and are thus an attractive therapeutic target[17]. In animals, overproduction or repeated infusion of the major protein in HDLs, apoA-I, reduces the extent of atherosclerosis[17], and two small clinical trials suggest that apoA-I has a similar effect in humans[18,19]. However, the 'HDL hypothesis' — that raising HDL concentrations has beneficial therapeutic effects — has been difficult to prove in humans because of the lack of interventions that substantially increase HDL-cholesterol concentrations. Some Japanese individuals have extremely high HDL concentrations, and the finding that this results from a genetic deficiency in the cholesteryl ester transfer protein (CETP) spurred the development of CETP inhibitors, which have been shown to raise HDL concentrations in humans[20]. However, the development of the first CETP inhibitor to advance into phase III clinical trials, torcetrapib, was terminated because of increased mortality and cardiovascular events[21]. Torcetrapib increases blood pressure and aldosterone concentrations through effects unrelated to inhibiting CETP, confounding the interpretation of the clinical trial and leaving hope for the development of a 'clean' CETP inhibitor[22]. Other approaches to increasing HDL concentration — such as inhibition of endothelial lipase (which breaks down HDLs) (Fig. 2) — are also of interest (Table 1). New targets could also be identified from ongoing studies of the molecular physiology of HDL metabolism and function, as well as from genome-wide association studies searching directly for genes that affect HDL concentrations (discussed later).

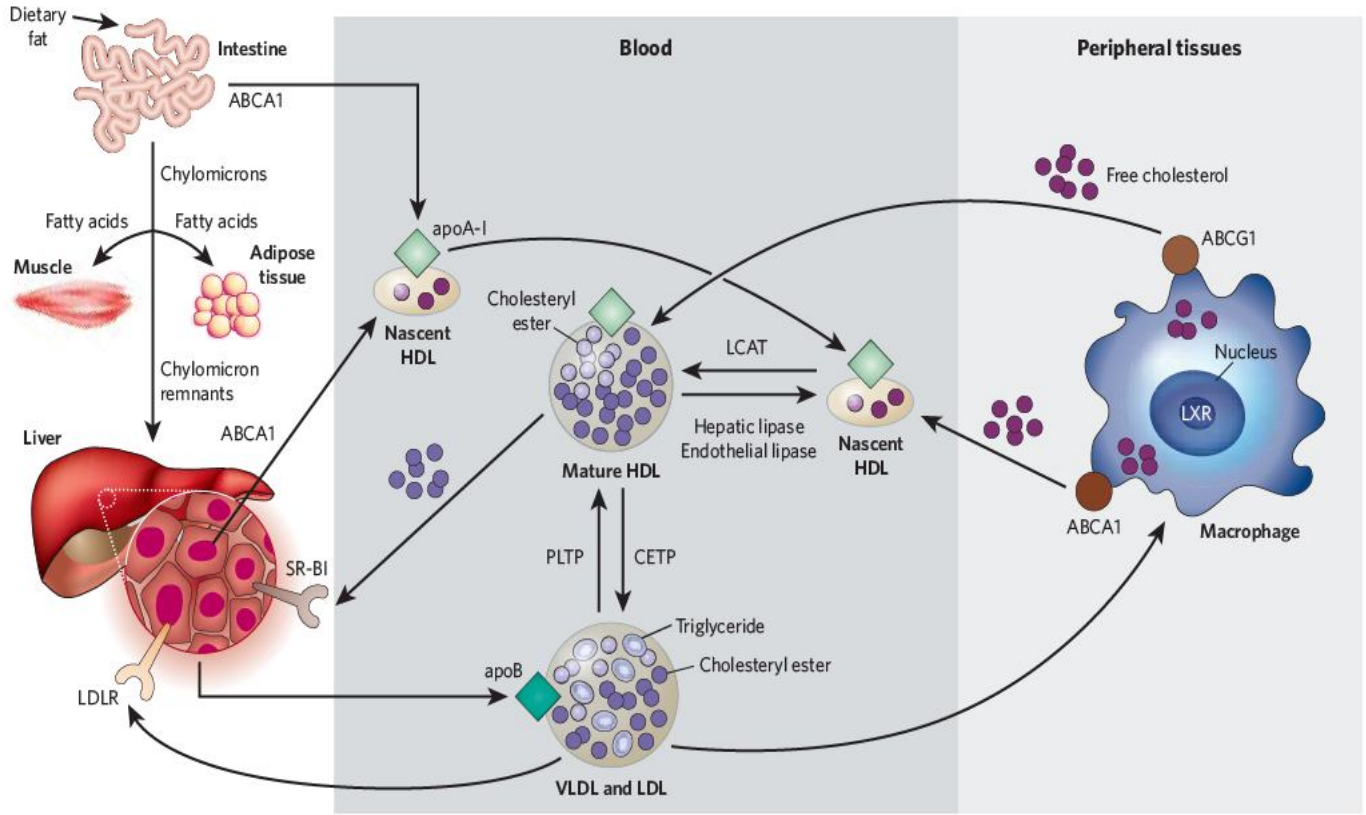

Figure 2 | Lipoprotein metabolism. Lipoprotein metabolism has a key role in atherogenesis. It involves the transport of lipids, particularly cholesterol and triglycerides, in the blood. The intestine absorbs dietary fat and packages it into chylomicrons (large triglyceride-rich lipoproteins), which are transported to peripheral tissues through the blood. In muscle and adipose tissues, the enzyme lipoprotein lipase breaks down chylomicrons, and fatty acids enter these tissues. The chylomicron remnants are subsequently taken up by the liver. The liver loads lipids onto apoB and secretes very-low-density lipoproteins (VLDLs), which undergo lipolysis by lipoprotein lipase to form low-density lipoproteins (LDLs). LDLs are then taken up by the liver through binding to the LDL receptor (LDLR), as well as through other pathways. By contrast, high-density lipoproteins (HDLs) are generated by the intestine and the liver through the secretion of lipid-free apoA-I. ApoA-I then recruits cholesterol from these organs through the actions of the transporter ABCA1, forming nascent HDLs, and this protects apoA-I from being rapidly degraded in the kidneys. In the peripheral tissues, nascent HDLs promote the efflux of cholesterol from tissues, including from macrophages, through the actions of ABCA1. Mature HDLs also promote this efflux but through the actions of ABCG1. (In macrophages, the nuclear receptor LXR upregulates the production of both ABCA1 and ABCG1.) The free (unesterified) cholesterol in nascent HDLs is esterified to cholesteryl ester by the enzyme lecithin cholesterol acyltransferase (LCAT), creating mature HDLs. The cholesterol in HDLs is returned to the liver both directly, through uptake by the receptor SR-BI, and indirectly, by transfer to LDLs and VLDLs through the cholesteryl ester transfer protein (CETP). The lipid content of HDLs is altered by the enzymes hepatic lipase and endothelial lipase and by the transfer proteins CETP and phospholipid transfer protein (PLTP), affecting HDL catabolism.

The experience with torcetrapib has focused increasing attention on new therapies that improve HDL function, as distinct from those that increase plasma HDL concentrations. The most well-established mechanism by which HDLs protect against atherosclerosis is by promoting cholesterol efflux from macrophages and transporting the cholesterol to the liver for excretion in bile and faeces, a process termed reverse cholesterol transport (Fig. 2). Although this process is difficult to quantify *in vivo*, circumstantial evidence supports the idea that reverse cholesterol transport contributes to HDL-mediated inhibition of atherogenesis. The molecular pathways of cholesterol efflux from macrophages and the molecular nature of the lipoprotein acceptor particles have been extensively investigated, as have the pathways by which cholesterol is returned to the liver for excretion. A seminal discovery was the identification of mutations in the gene encoding the transporter protein ABCA1 in individuals with Tangier disease[23]. ABCA1 promotes the efflux of cholesterol from cells, including macrophages, to lipid-poor apoA-I-containing particles[23], which are HDL precursors present in plasma. Cholesterol efflux from macrophages to mature HDLs was subsequently found to occur through a different transporter, ABCG1 (ref. 24). Both ABCA1 and ABCG1 have been shown to contribute to

Table 1 | Selected new therapeutic targets for atherosclerosis or its risk factors

Target	Human genetics*	Types of therapy	Biomarkers†	Phase‡
Lipoprotein metabolism				
Squalene synthase	None	Inhibitor	LDL cholesterol	III
MTP	Abetalipoproteinaemia	Inhibitor	LDL cholesterol, apoB	II/III
apoB	Hypobetalipoproteinaemia	Antisense oligonucleotide	LDL cholesterol, apoB	II
PCSK9	*PCSK9* gain of function and loss of function	Antisense oligonucleotide, small interfering RNA	LDL cholesterol	Preclinical
Thyroid-hormone receptor-β	None	Agonist	LDL cholesterol, triglycerides and HDL cholesterol	II
Farnesoid X receptor	None	Agonist	Triglycerides	I
		Antagonist	LDL cholesterol	Preclinical
Lipoprotein lipase	Familial chylomicronaemia, association	Gene-replacement therapy	Triglycerides	I/II
CETP	*CETP* deficiency, association	Inhibitor	LDL cholesterol, HDL cholesterol	II/III
Cannabinoid receptor 1	None	Antagonist	Triglycerides, HDL cholesterol and body weight	III
LXR	None	Agonist	*ABCA1* expression	I
Niacin receptor (GPR109A)	None	Agonist	Free fatty acids, triglycerides and HDL cholesterol	I
apoA-I	*APOA1* mutations	Full-length protein	apoA-I	I/II
		Mimetic peptides	Cholesterol efflux, anti-inflammatory function	I/II
		Upregulator	apoA-I	I
Lecithin cholesterol acyltransferase (LCAT)	*LCAT* deficiency	Agonist peptide	HDL cholesterol	II
Endothelial lipase	Association	Inhibitor	HDL cholesterol	Preclinical
Biologically active lipids				
TXA_2 receptor	None	Antagonist	TXA_2	Preclinical
mPGES1	None	Inhibitor	PGE_2	Preclinical
PGE_2 receptors (EP1 and EP3)	None	Antagonist	None	Preclinical
PGD_2 receptor (DP1)	None	Antagonist	Niacin-associated flushing	III
5-LO	Association	Inhibitor	LTB_4	Preclinical
FLAP	Association	Inhibitor	LTB_4	II
LTA_4 hydrolase	Association	Inhibitor	LTB_4	Preclinical
LTB_4 receptors (BLT1 and BLT2)	None	Antagonist	None	Preclinical
15-LO	None	Inhibitor	LTE_4	Preclinical
Myeloperoxidase	None	Inhibitor	Myeloperoxidase activity	Preclinical
Secretory PLA_2 (groups IIa, V and X)	Association	Inhibitor	Secretory PLA_2 activity	II
Lipoprotein-associated PLA_2	Association	Inhibitor	Lipoprotein-associated PLA_2 activity	II
Leukocyte recruitment and retention				
VCAM1	Association	Antagonist	Soluble VCAM1	Preclinical
ICAM1	Association	Antagonist	Soluble ICAM1	Preclinical
P selectin	Association	Inhibitor	Soluble P selectin	Preclinical
CD44	None	Inhibitor	Soluble CD44	Preclinical
CCR2	Association	Antagonist	None	Preclinical
CX_3CR1	Association	Antagonist	None	Preclinical
CCR7	None	Agonist	None	Preclinical
Extracellular-matrix turnover and plaque rupture				
MMPs (for example, MMP9)	Association	Inhibitor	MMP activity	Preclinical
Cathepsins (for example, cathepsin S)	None	Inhibitor	Cathepsin activity	Preclinical

*Human genetics refers to the existing data on mendelian disorders or candidate gene association that help to validate the target. †Biomarkers refers to the biomarker that might be used to determine efficacy and optimal dosing. ‡Phase refers to the current phase of drug development and is based on public domain information that can change rapidly.

the efflux of cholesterol from macrophages and to reverse cholesterol transport *in vivo*[25]. Transcription of the genes encoding ABCA1 and ABCG1 is stimulated by liver X receptor (LXR), a nuclear receptor that is activated by oxysterols (oxidized derivatives of cholesterol). Accordingly, LXR agonists promote cholesterol efflux from cultured macrophages[26], accelerate reverse cholesterol transport *in vivo*[27], and lead to substantial retardation or even regression of atherosclerosis in mice[26,28]. Thus, LXR is a target for the development of therapies focused on promoting reverse cholesterol transport to treat atherosclerotic disease (Table 1).

HDLs have various other properties that could contribute to their antiatherogenic properties. For example, HDLs can promote the activity of nitric-oxide synthase 3 (NOS3; also known as eNOS) and thereby increase the bioavailability of nitric oxide[29]. In addition, HDLs have anti-inflammatory effects both *in vitro* and *in vivo*, and these have been the subject of intensive study[30]. Intriguingly, the many activities of HDLs might have evolved from an original role in innate immunity. HDLs bind to lipopolysaccharide, a component of bacterial cell walls, and protect mice from lipopolysaccharide-induced mortality[31]. More recent work has shown that HDLs function as a platform for the assembly of a complex that contains apoL-I and haptoglobin-related protein and is highly lytic for a species of trypanosome[32]. Indeed, systematic proteomic analysis has revealed that a large number of proteins are bound to human HDLs, including proteins involved in inflammation, complement regulation and innate immunity[33], and variation in the protein composition of HDLs might affect HDL function. Protein components of HDLs have also been targeted as a therapy. For example, as indicated earlier, the therapeutic potential of peptides based on the sequence of apoA-I that can mimic the cholesterol-efflux-promoting and/or anti-inflammatory properties of HDLs has been pursued[34].

Inflammatory processes

Inflammation is crucial for the development of atherosclerotic plaques. Inflammatory pathways such as those involving biologically active lipids, the renin–angiotensin–aldosterone system and cellular processes within atherosclerotic plaques are involved in atherosclerosis, and components of these pathways are the targets of interventions (both in use and in development) for treating atherosclerotic cardiovascular disease.

Biologically active lipids

Biologically active lipids activate receptors — usually G-protein-coupled receptors — and, consequently, induce an intracellular signalling cascade. These lipids have been implicated in the pathogenesis of atherosclerosis, so the enzymes that generate them and the receptors that mediate their actions are attractive targets for therapy.

Prostaglandins are a family of biologically active lipids that are generated from arachidonic acid, which is present in the plasma membrane. The first step in prostaglandin synthesis is carried out by cyclooxygenase (COX), for which there are two distinct isozymes, COX1 and COX2. The cardioprotective effects of aspirin are thought to result from inhibition of COX1 in platelets, which reduces concentrations of the prothrombotic prostaglandin thromboxane A_2 (TXA_2). The efficacy of low-dose aspirin in the secondary prevention of myocardial infarction and stroke attests to the importance of prostaglandins in human cardiovascular disease. Findings that COX2-selective inhibitors have the opposite effect (that is, they increase the risk of atherothrombotic cardiovascular events[35]) indicate that prostaglandins have a highly complex role. These experiments in both mice and humans showed that COX2-selective inhibitors suppress the formation of another prostaglandin, prostacyclin (PGI_2), without affecting the COX1-mediated formation of TXA_2 (ref. 35). PGI_2 is atheroprotective in mice, and deletion of the gene encoding the PGI_2 receptor accelerates the development of atherosclerosis[36,37]. Conversely, TXA_2 is proatherogenic: deficiency or antagonism of the TXA_2 receptor results in reduced progression of atherosclerosis in mice[36]. Multiple mechanisms are likely to be involved in the effects of prostaglandins and their receptors on atherosclerosis, including control of not only platelet activation but also lipid peroxidation and leukocyte recruitment into the vessel wall. These findings[35–37] suggest that selective agonism of the PGI_2 receptor or antagonism of the TXA_2 receptor might have beneficial therapeutic effects for individuals with atherothrombotic disease.

Another prostaglandin, prostaglandin E_2 (PGE_2), is generated by several dedicated enzymes, including microsomal PGE synthase 1 (mPGES1; also known as PTGES). Experiments in mice suggest that mPGES1 promotes atherosclerosis: upregulation of expression of the gene encoding mPGES1 occurs in atherosclerosis, whereas deficiency in this gene results in reduced development of atherosclerosis, together with increased PGI_2 (but not TXA_2) biosynthesis (owing to diversion of the precursor PGH_2 from PGE_2 to PGI_2 generation)[38]. Thus, inhibiting mPGES1 could be a new approach to treating atherosclerosis. An alternative could be to block the activity of PGE_2 by preventing it from binding to its receptors. PGE_2 can activate four receptors: EP1, EP2, EP3 and EP4. In particular, antagonizing EP1 or EP3 — both of which are present at the cell surface of macrophages and have pro-inflammatory effects when activated — might have antiatherosclerotic effects.

PGD_2 is produced in atherosclerotic lesions by macrophages, as well as by mast cells (which can also be found in these lesions). The PGD_2 receptor (DP1) is expressed in the vasculature, but the effects of its activation on atherogenesis are unknown. An antagonist of DP1 has efficacy in reducing allergy-induced nasal congestion, as well as the cutaneous flushing associated with nicotinic acid (niacin) administration[39], and it is in clinical development for reducing niacin-associated flushing.

The leukotrienes are another family of biologically active lipids derived from arachidonic acid[40]. Leukotriene A_4 (LTA_4) is generated by the action of the enzyme 5-lipoxygenase (5-LO) with the aid of the 5-LO-activating protein (FLAP). LTA_4, in turn, is metabolized by the LTA_4 hydrolase to LTB_4. LTB_4 can then bind to and activate its receptors BLT1 and BLT2, which are expressed by vascular cells and leukocytes, promoting the recruitment of leukocytes into the vessel wall. Alternatively, LTA_4 can be conjugated to glutathione, yielding cysteinyl leukotrienes; these lipids bind to the receptors CysLT1 and CysLT2, which are also expressed by vascular cells and leukocytes. Both protein and lipid components of the 5-LO–LTB_4 pathway have been identified in atherosclerotic plaques in humans, and the concentrations of these components are higher in unstable plaques than in stable plaques[41]. In the past few years, genetic studies in humans have implicated the 5-LO–LTB_4 pathway in atherosclerotic cardiovascular disease. In a candidate gene association study, specific polymorphisms in the promoter of the gene *ALOX5AP* (which encodes FLAP) were found to be associated with variation in carotid intima-media thickness — a marker of atherosclerotic disease — and systemic markers of inflammation[42]. Furthermore, a study of large Icelandic pedigrees showed linkage of *ALOX5AP* to risk of myocardial infarction. This finding was replicated in other cohorts, in which a particular haplotype of this gene was found to be associated with a twofold increase in the risk of myocardial infarction and stroke[43]. Further investigation of candidate genes in the 5-LO pathway yielded evidence that certain polymorphisms in the gene encoding LTA_4 hydrolase were significantly associated with myocardial-infarction risk, an effect that was particularly notable in individuals of African descent[44]. In mice, an early study suggested that deficiency in 5-LO reduces the development of atherosclerosis in mice[45]; however, this conclusion was not supported by a subsequent study[46]. In addition, antagonism of the LTB_4 receptors BLT1 and BLT2, or deletion of the genes encoding these, was reported to reduce atherosclerosis in mice[40]. Thus, inhibition of the 5-LO–LTB_4 pathway could be a therapeutic approach to atherosclerosis. Indeed, a FLAP inhibitor has entered clinical development for the treatment of atherosclerotic cardiovascular disease[47].

The unsaturated fatty acid at the *sn*-2 position of phospholipids is prone to oxidation in the arterial intima, and a growing body of evidence indicates that the resultant oxidized phospholipids are highly pro-inflammatory and contribute to atherogenesis[48]. There is substantial interest in identifying the specific enzymes — such as lipoxygenases, NADPH oxidases and myeloperoxidase — that create the environment of increased oxidant stress that promotes lipid peroxidation. Accumulating data suggest that myeloperoxidase is an important

enzymatic catalyst of lipid peroxidation, particularly at sites of inflammation such as the atherosclerotic lesion[49]. Myeloperoxidase also catalyses reactions that modify proteins (such as nitration, halogenation and carbamoylation[50]) and can influence protein function and promote atherogenesis. Phospholipases that cleave parent and oxidized phospholipids within the atherosclerotic plaque might also contribute to inflammation and therefore could be targets for inhibition. The family of secretory phospholipase A_2 (PLA_2) enzymes (particularly group IIa, group V and group X) has been implicated in atherogenesis[51]. Lipoprotein-associated PLA_2 might also contribute to inflammation; this enzyme cleaves oxidized phospholipids that have a short-chain oxidized fatty acid at the *sn*-2 position (for example, platelet-activating factor) and might generate pro-inflammatory and proatherogenic products[52]. Inhibition of secretory PLA_2 enzymes and lipoprotein-associated PLA_2 is actively being pursued, with compounds targeting these enzymes in clinical development (Table 1).

Oxidized phospholipids can also elicit a beneficial immune response, as shown by a study in which immunization of atherosclerotic mice with oxidized forms of LDL generated antibodies that reduced the size of lesions[53]. This beneficial response was found to be mediated by the antibody T15 (which is a 'natural' antibody', because it can be produced in the absence of immunization); T15 recognizes the head group of oxidized phospholipids and inhibits the accumulation of modified lipoproteins in macrophages. Immunization with oxidized forms of LDL leads to increased production of interleukin-5 (IL-5), which stimulates B1 cells to secrete T15 (ref. 54). In humans, plasma concentrations of oxidized phospholipids, as determined by binding to T15, are strongly correlated with plasma concentrations of lipoprotein (*a*) — an independent cardiovascular risk factor of unknown function — and are independently predictive of angiographic coronary disease[55]. The implications of these findings for the development of new therapies have yet to be determined.

Renin–angiotensin–aldosterone system

The renin–angiotensin–aldosterone system, long recognized to be a crucial regulator of blood pressure, has consistently been shown to have a prominent role in atherogenesis in both humans and experimental animals. Effective therapies for atherosclerotic cardiovascular disease based on inhibition of this system were developed from research in multiple fields, similar to the development of statins. Several lines of evidence indicate that the proatherogenic effects of activating this system are not solely the result of increases in blood pressure. Chronic infusion of angiotensin II promotes atherosclerosis in hyperlipidaemic mice independently of changes in arterial blood pressure[56,57]. Conversely, in a wide range of experimental animals, pharmacological inhibition or genetic deficiency of components of the renin–angiotensin–aldosterone system effectively reduces the development of atherosclerosis independently of blood-pressure reduction[58]. Many inhibitors of the renin–angiotensin–aldosterone system are clinically approved for reducing blood pressure, including drugs that target renin (which catalyses the first step in the pathway, cleavage of angiotensinogen to generate angiotensin I), angiotensin-I-converting enzyme (ACE, which converts angiotensin I into angiotensin II), the AT1 receptors (receptors for angiotensin II), and the mineralocorticoid receptor (the receptor for aldosterone). In humans, ACE inhibitors have a beneficial effect on atherosclerotic disease, even in individuals who do not have high blood pressure[59]. In addition to angiotensin II, a family of biologically active angiotensin-I-derived peptides has recently been identified; these peptides have a broad range of actions on all of the main cell types in atherosclerotic lesions. Further understanding of the mechanisms and consequences of the actions of these peptides could lead to the identification of new therapeutic targets.

Cellular processes in atherosclerotic lesions

Endothelial cells form a continuous monolayer on the luminal surface of atherosclerotic lesions. Hence, these cells have a crucial role in the recruitment and adhesion of leukocytes, whose infiltration into lesions is a prominent feature of atherosclerosis (Fig. 3). Knowledge of the molecular mechanisms that lead to leukocyte recruitment is continually being refined[60]. Endothelial cells display several adhesion molecules at the cell surface, and a deficiency in these molecules has been shown to decrease lesion formation in mice. Deficiency in vascular cell-adhesion molecule 1 (VCAM1) has the most marked effects, whereas deficiency in intercellular adhesion molecule 1 (ICAM1) or platelet selectin (P selectin) has less of an effect[61]. In addition, expression of the gene encoding the adhesion molecule CD44 is upregulated specifically in atherosclerotic lesions, and deletion of this gene results in reduced monocyte recruitment and less development of atherosclerosis[62]. Thus, blockade of one or more key adhesion molecules might be an effective strategy to reduce lesion formation.

An important property of endothelial cells is their ability to sense changes in vascular flow dynamics[63]. In normal (laminar) flow conditions, endothelial cells produce small amounts of adhesion molecules, but production of these is increased in non-laminar or turbulent flow. There has been substantial interest in understanding the molecular mechanisms by which these flow changes are sensed and trigger changes in intracellular signalling and gene expression. Recent findings suggest that Kruppel-like factor 2 has a central role in endothelial mechanotransduction, by regulating the transcriptional response to changes in flow dynamics[64]. Kruppel-like factor 2 affects the expression of a wide variety of genes involved in atherosclerotic lesion development and is therefore a plausible target for therapy.

The recruitment of monocytes to the intima and their differentiation into macrophages are the primary cellular events during the initiation of a lesion, and this recruitment continues during the expansion and progression of the atherosclerotic lesion (Fig. 3). Numerous mediators that attract monocytes to developing lesions have been identified. One of the early steps in this process is the binding of the chemoattractant cytokine (chemokine) CCL2 to its receptor (CCR2). Recent studies have defined distinct subsets of monocytes that are preferentially recruited to lesions[65,66]. Using an antibody directed against cell-surface molecules called Ly6 antigens, a subset of monocytes was found to accumulate preferentially in atherosclerotic lesions: these cells display large amounts of Ly6C at the cell surface, as well as the chemokine receptors CCR2 and CX_3CR1. Thus, it might be possible to inhibit selectively the recruitment of discrete monocyte subpopulations into the arterial wall, avoiding the potentially adverse consequences of broadly inhibiting monocyte recruitment.

Within the intima of the atherosclerotic lesion, monocyte-derived macrophages have many activities, including intracellular accumulation of lipids and secretion of (potentially) a wide range of chemokines, other cytokines and proteases (Fig. 3). Like the monocytes they are derived from, macrophages are heterogeneous[67]. In the presence of cytokines such as interferon-γ, tumour-necrosis factor (TNF) and granulocyte–macrophage colony-stimulating factor, macrophages are activated through the classical pathway; this class of activated macrophage (sometimes referred to as M1 cells) produces inducible nitric-oxide synthase and secretes IL-1β, IL-6 and TNF. By contrast, in the presence of various other stimuli, including IL-4 and IL-13, macrophages are activated through the alternative pathway; this class of activated macrophage (sometimes referred to as M2 cells) assists in resolving inflammation through increased endocytic activity, which is mediated by the class A scavenger receptor (also known as MSR1) and the macrophage mannose receptors[68]. The relative importance of different macrophage classes in atherogenesis is, however, uncertain at present.

Macrophage function is regulated by Toll-like receptors (TLRs), which are pattern-recognition receptors that are involved in initiating innate immune responses. The genes encoding several TLRs — including TLR1, TLR2, TLR4 and TLR5 — are expressed in atherosclerotic lesions. Hyperlipidaemic mice that are deficient in TLR2 or TLR4 (refs 69, 70) — or in the main TLR adaptor protein, MyD88 (refs 69, 71) — have smaller atherosclerotic lesions. Modified lipids or heat-shock proteins that are present in lesions have been suggested to function as endogenous ligands for TLRs[72]. In addition, SNP-based association

studies have provided evidence that TLRs are important in human atherosclerosis[73]. But whether targeting TLRs is a viable approach for treating atherosclerosis remains to be determined.

The role of leukocytes other than monocytes and macrophages in atherosclerosis continues to be debated[74]. Although there is a substantial body of research on the involvement of lymphocytes, the complexities of these cells have confounded a definition of their precise roles[75]. Recent studies have begun to investigate how natural killer cells[74], mast cells[76] and platelets[77] might also contribute to atherosclerosis. Of particular interest is the finding that dendritic cells can exit from lesions (Fig. 3); this egress depends on CCR7 signalling and might promote lesion regression, through the removal of lipid from the lesion[78,79]. These findings provide a new model for how lesion size can be modulated and indicate that activation of CCR7 could promote regression of atherosclerosis.

Another area of intense recent interest concerns the possibility that bone-marrow-derived endothelial and smooth muscle progenitor cells are recruited to lesions, raising the exciting possibility that a cell-based approach could be used to treat atherosclerosis. Some evidence suggests that the recruitment of these cells contributes considerably to atherosclerotic lesion formation[80]. However, this concept is controversial, with some studies finding large numbers of bone-marrow-derived endothelial cells and smooth muscle cells in atherosclerotic lesions in experimental models[81], and others finding only a small number of such cells[82].

Atherosclerosis-associated thrombosis

Atherosclerotic lesions trigger acute cardiovascular disease such as myocardial infarction and stroke only when an occlusive thrombus (or clot) forms (see page 914). The formation of a thrombus has been ascribed to at least two different types of event: first, rupture of the surface of the lesion, exposing a thrombogenic subendothelial layer of the blood vessel; and second, erosion of the fibrous cap of the lesion (which consists of smooth muscle cells)[83]. Elucidating how lesions rupture or erode and testing interventions that could prevent these events would be greatly assisted by using an animal model. However, there is no widely accepted animal model. Ruptured atherosclerotic lesions have been found in apoE-deficient mice, but the extent to which this pathology reproduces that responsible for acute cardiovascular events in humans is a matter of intense debate[84,85]. In addition, mice deficient in both apoE and the class B scavenger receptor SR-BI have accelerated atherosclerosis and spontaneous myocardial infarctions[86]; however, it is unclear whether these infarctions are caused by thrombi that form as a consequence of atherosclerosis.

Elucidating the mechanisms underlying plaque rupture has been the main focus of research on atherosclerosis-associated thrombosis. Plaque rupture is associated with the degradation of the extracellular-matrix components collagen and elastin, so it is logical to infer that extracellular proteases are involved in this process. Many members of the matrix metalloproteinase (MMP) class of enzymes have been found in atherosclerotic plaques and have been suggested to be involved in rupture (see ref. 87 for a review). Most of these studies showed increased production of specific MMPs at the shoulder regions of lesions, where plaques usually rupture. MMP9, which is produced by macrophages, is the most commonly detected MMP. This enzyme is functionally important for plaque rupture in mice: expression of a gene encoding an activated form of MMP9 in apoE-deficient mice results in the disruption of atherosclerotic lesions in the brachiocephalic artery[88]. Another class of proteases, cathepsins, has also been suggested to contribute to lesion disruption. Although cathepsins are lysosomal proteases, which function in the acidic conditions of the lysosome, they maintain the ability to degrade elastin and collagen in the extracellular environment, which is pH neutral. In mice, deficiency in particular types of cathepsin results in an increased abundance of extracellular-matrix components, and this is thought to decrease the propensity of plaques to rupture[89].

The death of cells in atherosclerotic lesions is thought to increase the risk of plaque rupture because, at late stages of disease, increased leukocyte recruitment to plaques is associated with increased levels of necrosis and apoptosis[90]. Studies in mice have shown that the apoptosis of macrophages in atherosclerotic lesions is triggered by endoplasmic-reticulum stress and

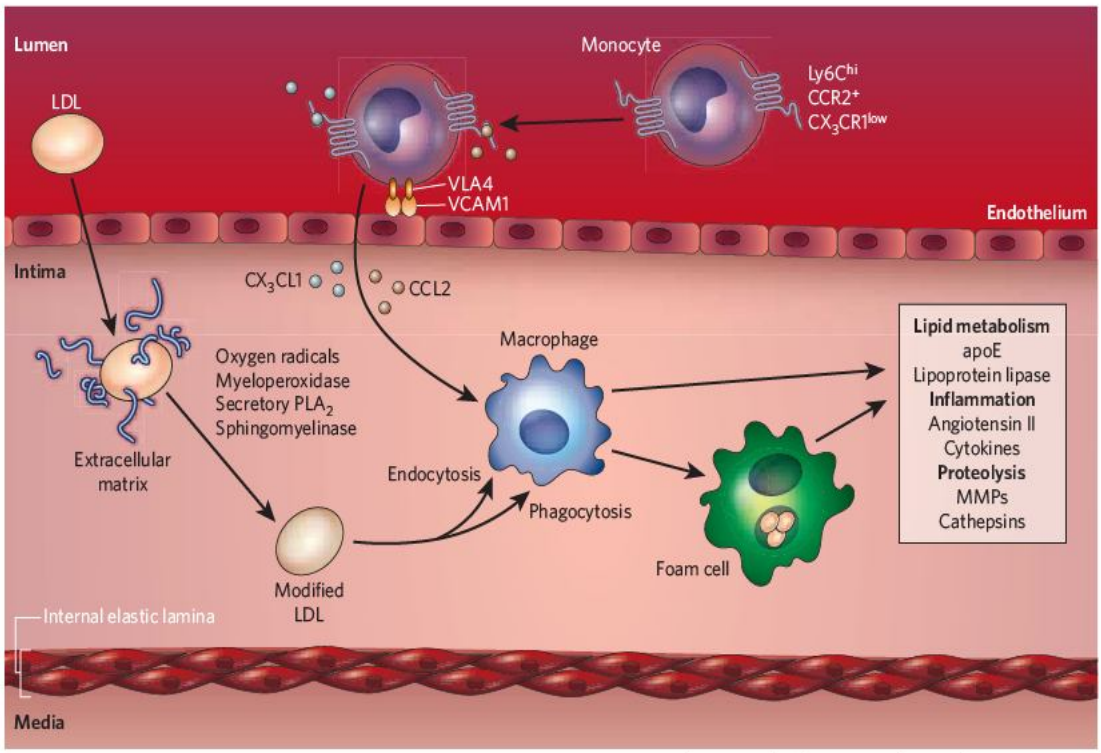

Figure 3 | Recruitment of monocytes and formation of foam cells. LDLs in the blood enter the intima, where they are retained through binding to the extracellular matrix. LDLs are then modified by oxygen radicals, myeloperoxidase, secretory phospholipase A_2 and sphingomyelinase. This results in the generation of pro-inflammatory biologically active lipids that initiate and maintain an active inflammatory process in the intima (not shown). This inflammation results in the generation of chemokines such as CX_3CL1 and CCL2, which recruit subsets of monocytes to the intima. These monocytes then differentiate into macrophages, which take up modified LDL through endocytosis or phagocytosis and become foam cells (which are loaded with cholesterol). Macrophages secrete various factors involved in propagating the atherosclerotic plaque, including factors involved in lipid metabolism, inflammation and proteolysis. VLA4, very late activation protein 4 (also known as $\alpha_4\beta_1$-integrin).

signalling through multiple receptors, including the class A macrophage scavenger receptor and TLR4, leading to Jun amino-terminal kinase (JNK)-dependent apoptosis[91]. The death of vascular smooth muscle cells might also contribute to rupture. For example, the induction of apoptosis specifically in vascular smooth muscle cells in apoE-deficient mice has no effect on atherosclerotic lesion size but affects the cellular composition of lesions, and such changes in lesion composition are expected to make the lesions more prone to rupture[92]. The role of cell death in atherogenesis remains poorly defined at all stages of lesion development and is an important area for future investigation.

Genetics of atherosclerosis in humans

Although animal models of atherosclerosis provide valuable information, their relevance to human disease remains uncertain. The current rapid progress in human genetic studies could, however, provide unprecedented mechanistic insight into human pathophysiology.

Atherosclerotic cardiovascular disease is clustered in families and has a strong genetic component, but identifying the genes that contribute to risk, beyond those affecting the 'traditional' risk factors, has been difficult. Linkage studies in large pedigrees with premature coronary artery disease have generally failed to identify causal genes definitively, with rare exceptions such as *LRP6*. By contrast, genome-wide association studies have emerged as a potentially powerful method of identifying genes underlying complex traits such as coronary artery disease. A striking and consistent finding is the highly significant association of a locus on chromosome 9p21 with myocardial infarction or coronary artery disease[93–95]. The risk-associated allele is common, with a frequency of almost 50%, and each copy of this allele increases the risk of myocardial infarction by about 25%. The genes nearest to the SNPs that are most highly associated with risk encode cyclin-dependent kinase inhibitors: *CDKN2A* (which encodes INK4A) and *CDKN2B* (which encodes INK4B). The encoded proteins are members of the INK4 family of cell-cycle suppressors, which regulates the G1–S cell-cycle checkpoint and has a role in transforming growth factor-β (TGF-β)-mediated growth inhibition, a process implicated in the pathogenesis of atherosclerosis[96]. The roles of *CDKN2A* and *CDK2NB* in atherogenesis remain to be determined, but these genetic findings[93–95] strongly implicate cell-cycle regulation in the pathogenesis of atherosclerotic cardiovascular disease.

A genome-wide association study has also implicated genes involved in cell proliferation in the risk of myocardial infarction[95]. This study presented convincing evidence that three genes (*PSRC1*, *MIA3* and *SMAD3*) encoding cell-growth regulators are significantly associated with myocardial-infarction risk. Notably, SMAD3 is an intracellular signalling molecule that links activation of the TGF-β receptor to transcriptional regulation. This study also identified a locus near the gene encoding the chemokine CXCL12 as significantly associated with myocardial-infarction risk. This chemokine was originally thought to regulate the homing of haematopoietic stem cells to the bone marrow and is now recognized to have a role in the mobilization, homing and differentiation of vascular progenitor cells in response to vascular injury[97]. Another locus identified in this study is near the gene encoding methylenetetrahydrofolate-dehydrogenase-1-like protein (the mitochondrial C1-tetrahydrofolate synthase), but how this protein might affect myocardial-infarction risk is unclear.

Genome-wide association studies can also be used to probe the genetic basis of risk factors for atherosclerosis, such as type 2 diabetes, high blood pressure and dyslipidaemia[98]. The Diabetes Genetics Initiative, for example, was designed to investigate type 2 diabetes, but 18 other phenotypes, including plasma lipid concentrations, were analysed as secondary traits[99]. Several of the loci that are significantly associated with changes in lipid concentrations are in or near genes in which mutations have been shown to cause mendelian syndromes affecting lipid concentrations (namely genes that encode the proteins apoE, ABCA1, apoA-V, CETP, lipoprotein lipase and hepatic lipase). However, other associations were also uncovered; for example, a SNP in the gene *GCKR*, which encodes glucokinase regulatory protein, was found to have a highly significant association with triglyceride concentrations[99]. This protein regulates glucokinase, the first enzyme in the glycolytic pathway, and it can affect hepatic triglyceride synthesis. The subsequent analysis, accompanied by extensive replication of results, of three genome-wide association studies for type 2 diabetes[100] found that loci near the genes *ANGPTL3* (which encodes angiopoietin-like 3, a protein that affects triglyceride metabolism in mice) and *MLXIPL* (which encodes carbohydrate-response-element-binding protein, a transcription factor that connects hepatic carbohydrate flux with fatty-acid synthesis) are significantly associated with triglyceride concentrations. In addition, loci on chromosome 1p13 (near the genes *CELSR2, PSRC1* and *SORT1*), 19p13 (near *CILP2* and *PBX4*) and 8q24 (near *TRIB1*) were found to be significantly associated with LDL-cholesterol concentrations. The locus on 1p13 is noteworthy because a genome-wide association study of myocardial infarction also identified this locus as being significantly associated with myocardial infarction[95]. *SORT1* encodes the protein sortilin 1, a multi-ligand cell-surface receptor, which could plausibly affect lipoprotein metabolism and thus atherosclerosis. Finally, the gene *GALNT2* — which encodes *N*-acetylgalactosaminyltransferase 2, an enzyme involved in O-linked glycosylation — was found to be significantly associated with HDL-cholesterol concentrations. Thus, genome-wide association approaches are beginning to yield new biological insights and potential therapeutic targets for atherosclerosis and its risk factors.

Perspectives

Atherosclerosis has been the subject of an immense amount of basic and applied research, resulting in substantial advances in understanding the molecular pathogenesis of the disease. These insights have led to the development of successful therapeutic interventions for atherosclerotic cardiovascular disease; for example, reducing plasma concentrations of atherogenic lipoproteins (notably with statins) and blocking renin–angiotensin–aldosterone system activity (notably with ACE inhibitors and angiotensin-receptor blockers). Results from studies in cell biology, whole-animal physiology, human genetics and mechanism-based research in humans will need to be integrated carefully to choose the next generation of therapeutic targets. LDL-cholesterol concentrations and blood pressure are likely to continue to be acceptable surrogate end points in clinical trials for the registration of new drugs; however, the registration of new therapies for which the efficacy cannot be tracked by these end points will require proof of efficacy in morbidity and mortality trials. Decisions regarding which drugs and which doses to advance into large clinical end-point trials will be difficult, and new biomarkers (see page 949) and non-invasive imaging modalities (see page 953) will be needed to improve this process. Ultimately, the huge investment of the biomedical community in multidisciplinary research in atherosclerosis is likely to pay major dividends with the development of a new generation of therapies, ranging from those that can prevent disease to those that can cause its regression. ■

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Triggers, targets and treatments for thrombosis

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Thrombosis — localized clotting of the blood — can occur in the arterial or the venous circulation and has a major medical impact. Acute arterial thrombosis is the proximal cause of most cases of myocardial infarction (heart attack) and of about 80% of strokes, collectively the most common cause of death in the developed world. Venous thromboembolism is the third leading cause of cardiovascular-associated death. The pathogenic changes that occur in the blood vessel wall and in the blood itself resulting in thrombosis are not fully understood. Understanding these processes is crucial for developing safer and more effective antithrombotic drugs.

The pathophysiology of arterial thrombosis differs from that of venous thrombosis, as reflected by the different ways in which they are treated. In broad terms, arterial thrombosis is treated with drugs that target platelets, and venous thrombosis is treated with drugs that target proteins of the coagulation cascade. The available antithrombotic drugs are effective at reducing arterial thrombosis and venous thrombosis in patients with cardiovascular disease. However, the main side effect of these drugs is bleeding, which limits their use. To develop a new generation of safe and effective antithrombotic drugs with larger therapeutic windows (that is, a larger difference between the dose that prevents thrombosis and the dose that induces bleeding), a better understanding of the pathogenic processes that lead to thrombotic occlusion of blood vessels is needed. In this article I describe the pathological mechanisms and the risk factors that are known to lead to arterial thrombosis and venous thrombosis, and discuss the development of new approaches for antithrombotic therapy.

Arterial thrombosis

The primary trigger for arterial thrombosis is the rupture of an atherosclerotic plaque (Fig. 1a), which develops through the accumulation of lipid deposits and lipid-laden macrophages (foam cells) in the artery wall (see page 904). The thrombi that form at ruptured plaques are rich in platelets, which are small (about 1 μm in diameter) anucleate cells produced by megakaryocytes in the bone marrow[1]. These disc-shaped cells circulate in the blood as sentinels of vascular integrity and rapidly form a primary haemostatic plug at sites of vascular injury[2]. When an atherosclerotic plaque ruptures, platelets are rapidly recruited to the site, through the interaction of specific platelet cell-surface receptors with collagen and von Willebrand factor[3,4] (Fig. 2). After this adhesion to the vessel wall, the receptor-mediated binding of additional platelets (termed platelet aggregation) then results in rapid growth of the thrombus. Platelets also become activated at this stage. A major pathway of activation involves the cleavage and, consequently, the activation of the platelet receptor PAR1 (protease-activated receptor 1; also known as the thrombin receptor) by the protease thrombin (also known as factor II)[5], which is activated by the blood coagulation cascade. Activated platelets then release the contents of granules, which further promote platelet recruitment, adhesion, aggregation and activation.

The coagulation cascade (Fig. 3) is the sequential process by which coagulation factors of the blood interact and are activated, ultimately generating fibrin, the main protein component of the thrombus, and this cascade operates in both arterial and venous thrombosis. The cascade

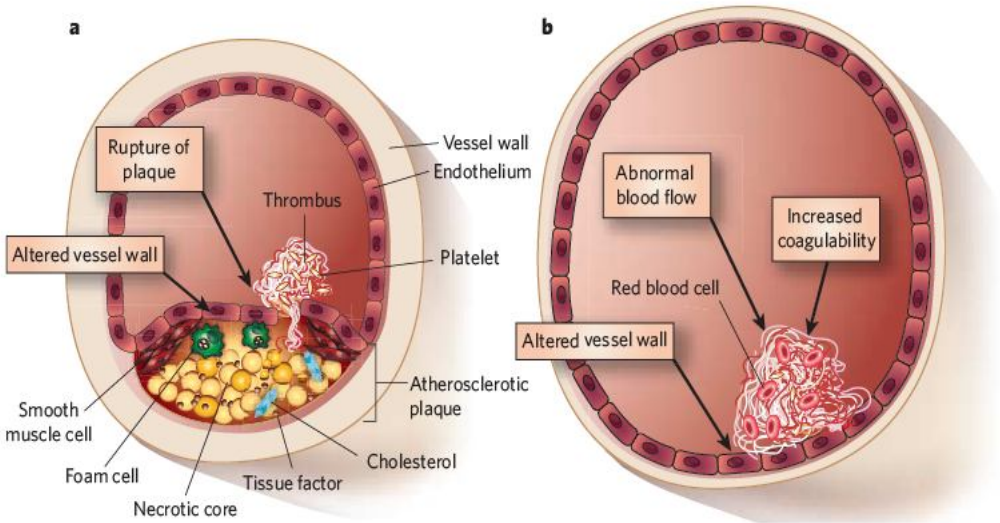

Figure 1 | Triggers of arterial and venous thrombosis. a, Artery. The primary trigger of arterial thrombosis is rupture of an atherosclerotic plaque. This involves disruption of the endothelium and release of constituents of the plaque into the lumen of the blood vessel. **b**, Vein. By contrast, in venous thrombosis, the endothelium remains intact but can be converted from a surface with anticoagulant properties to one with procoagulant properties. Venous thrombosis can be triggered by several factors: abnormal blood flow (such as the absence of blood flow); altered properties of the blood itself (thrombophilia); and alterations in the endothelium.

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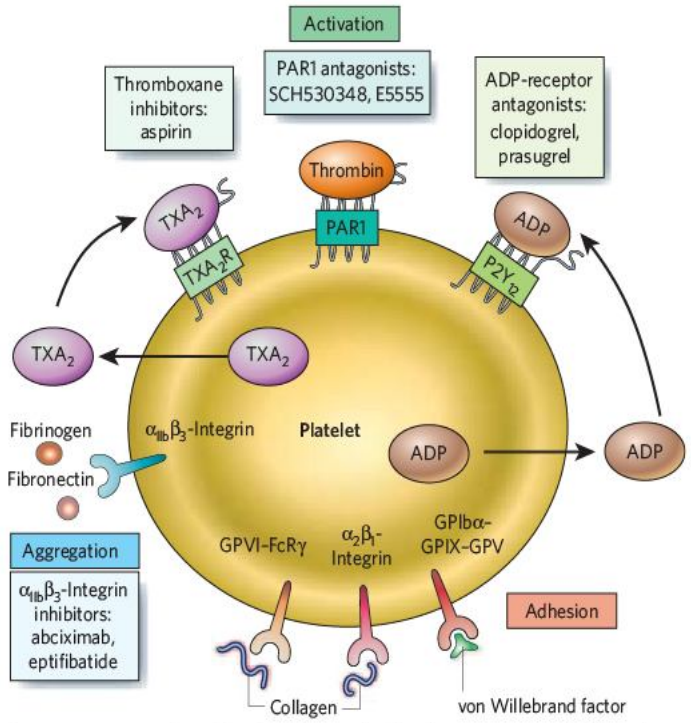

Figure 2 | Targets of antiplatelet drugs. Platelets have a variety of cell-surface receptors that mediate their activation (green shading), their adhesion to the blood vessel wall (red) and their aggregation with each other (blue). The ligands for various receptors are shown. Antiplatelet drugs and their targets are also indicated; targets include thromboxane A_2 (TXA_2), protease-activated receptor 1 (PAR1), the ADP receptor $P2Y_{12}$ and $\alpha_{IIb}\beta_3$-integrin.

is initiated by exposure of the blood to tissue factor (also known as factor III), a protein that is present at high concentrations in atherosclerotic plaques[6,7]. Circulating tissue factor is also present at increased concentrations in patients with cardiovascular disease and might contribute to thrombosis after plaque rupture[8,9].

In the case of acute thrombotic events, drugs that reduce the growth of a thrombus can be administered; the main target of these drugs is platelets. Antiplatelet drugs are also used prophylactically to reduce the incidence of arterial thrombosis in patients with cardiovascular disease[10]. The primary targets of antiplatelet therapy are molecules involved in platelet activation and aggregation (Box 1). At present, there are no drugs in clinical use that block the binding of platelets to collagen and von Willebrand factor and hence their adhesion to the blood vessel wall. In theory, inhibition of this early step in thrombus formation is more likely to disrupt the primary role of platelets in normal blood clotting (haemostasis) and therefore to increase the risk of bleeding. Nevertheless, such inhibitors are in development[10–12].

Another important treatment for acute thrombotic events is the degradation of fibrin, which stabilizes the structure of a thrombus[13], by using activators of the fibrinolytic system: namely 'clot busters', such as tissue plasminogen activator and streptokinase. However, the success of such treatment depends crucially on the timing of intervention, with earlier intervention generally having a better outcome. For example, for acute myocardial infarction, fibrinolytic therapy seems to be beneficial for at least 12 hours after the onset of symptoms. By contrast, fibrinolytic therapy for stroke has proven beneficial only when used within 3 hours[14] and can have the side effect of inducing brain haemorrhage. Therefore, researchers are focusing on strategies that protect the vasculature but have a lower incidence of brain haemorrhage than is induced by current fibrinolytic therapy.

In the past few years, studies identifying platelet receptors and signalling mechanisms have yielded a trove of new targets for antiplatelet therapy. For example, recent studies have shown that several cell-surface receptor–ligand interactions occur on close contact between platelets, such as the binding of the ligand semaphorin 4D to its receptors, CD72 and plexin B1 (ref. 15). These receptors mediate platelet–platelet interactions and thrombus retraction and hence are attractive therapeutic targets. Another example is the receptor CD36. It is well established that CD36 functions as a scavenger receptor at the surface of macrophages: it binds to oxidized low-density lipoproteins, resulting in the formation of foam cells and therefore contributing to the development of atherosclerotic plaques[16]. CD36 is also present at the surface of platelets. A recent study showed that oxidized low-density lipoproteins activate platelets by binding to CD36 and that the prothrombotic phenotype of mice deficient in apolipoprotein E, which have high concentrations of low-density lipoprotein in the

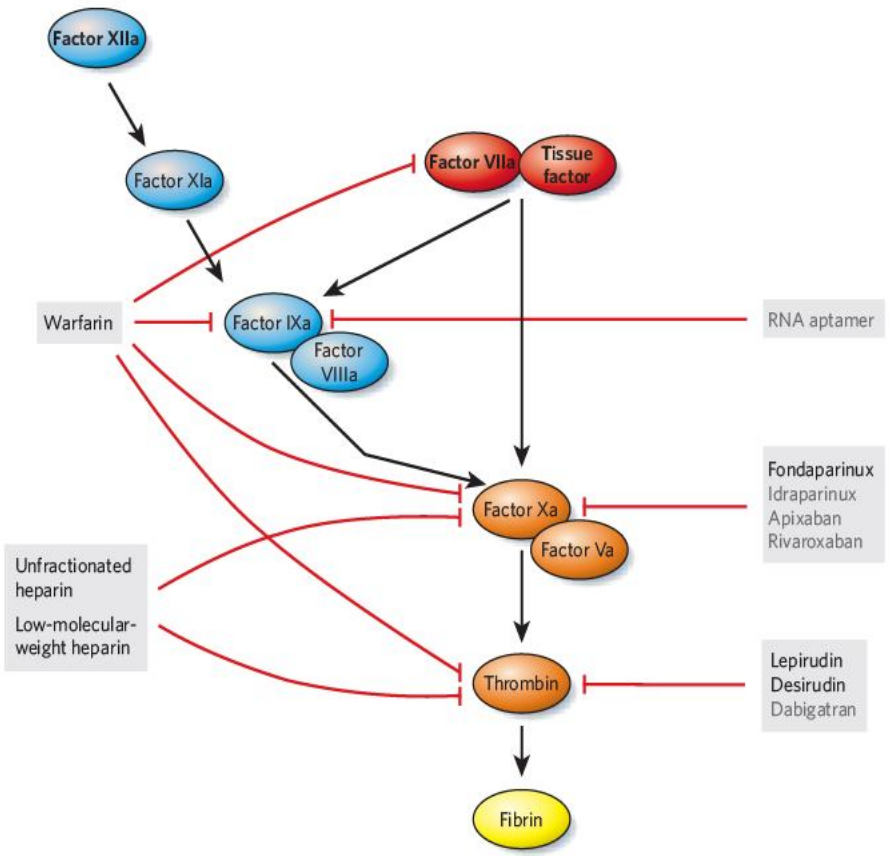

Figure 3 | Targets of anticoagulant drugs. Tissue factor is present at high concentrations in atherosclerotic plaques. When exposed to the blood — for example, when a plaque ruptures — tissue factor binds to the plasma protein factor VIIa (the extrinsic pathway, red), and this complex triggers activation of the coagulation cascade through the proteolytic cleavage of both factor X and factor IX. This cascade ultimately generates fibrin (through the common pathway, orange), which (on polymerization) stabilizes platelet thrombi. The coagulation cascade is amplified by the tenase complex, which consists of factor VIIIa and factor IXa (components of the intrinsic pathway, blue). Factor XIa and factor XIIa might also help to activate the coagulation cascade under pathological conditions. Triggers of thrombosis are shown in bold face. Anticoagulant drugs that are in current use (black) and in development (grey) are listed, and their targets are also indicated (red blocking arrows).

circulation, is reduced by deletion of *Cd36* (ref. 17), suggesting that this interaction could explain the increased platelet reactivity and thrombosis associated with hyperlipidaemia. Finally, the complexities of 'outside-in' and 'inside-out' signalling in platelets (that is, signalling that originates extracellularly or intracellularly, respectively) have begun to be unravelled. However, redundancy in signalling pathways makes it difficult to identify therapeutic targets. An exception seems to be the essential role of the cytoskeletal protein talin 1 (ref. 18). The binding of talin 1 to the cytoplasmic domain of β_3-integrin was shown to be required for activation of $\alpha_{IIb}\beta_3$-integrin (also known as glycoprotein IIB (GPIIB)–GPIIIA)[19]. Moreover, changing a single amino acid in the cytoplasmic domain of β_3-integrin selectively disrupted talin-1 binding and reduced arterial thrombosis in an animal model[19], suggesting that blockade of this interaction could be a new antithrombotic strategy.

Box 1 | Antiplatelet therapy

Antiplatelet drugs are used for both the prevention and the acute treatment of arterial thrombosis. These drugs target the activation and the aggregation of platelets. The advantages and disadvantages of the main types of antiplatelet drug are described below.

Cyclooxygenase inhibitors

Aspirin has been used clinically for more than 40 years and is the most commonly used antiplatelet drug. It inhibits platelet cyclooxygenase 1, which is required for the synthesis of thromboxane A_2 (TXA_2), a potent activator of platelets (Fig. 2). Aspirin not only significantly reduces the incidence of a first myocardial infarction in men at risk of cardiovascular disease (primary prevention) but also reduces the risk in patients who have had a myocardial infarction (secondary prevention)[46,47]. Aspirin therapy, however, is not without risk and can cause stomach ulcers and bleeding.

Recent studies of the selective cyclooxygenase-2 inhibitors rofecoxib and valdecoxib have shown that inhibiting the 'wrong' cyclooxygenase can lead to a significant increase in the incidence of myocardial infarction and stroke, resulting in the withdrawal of these drugs from the market[48]. It has been proposed that these inhibitors reduce cyclooxygenase-2-dependent synthesis of prostacyclin (also known as PGI_2), an inhibitor of platelet activation[48].

ADP-receptor antagonists

Another class of antiplatelet drug targets the ADP receptor $P2Y_{12}$, also reducing platelet activation[49] (Fig. 2). Clopidogrel, the most widely used drug in this class, is used to treat patients with acute coronary syndromes (which include various conditions, such as unstable angina, that are associated with chest pain as a result of reduced blood supply to the heart)[50,51]. Clopidogrel is also administered to those undergoing percutaneous coronary intervention (a procedure in which blood flow is restored to a coronary artery by using a catheter to remove an occlusive atherosclerotic plaque and position a stent). A recent study compared clopidogrel with another $P2Y_{12}$ antagonist, prasugrel, for the treatment of thrombosis in patients with acute coronary syndromes who are undergoing percutaneous coronary intervention[23]. Prasugrel significantly reduced the rate of ischaemic events (12.1% for clopidogrel versus 9.9% for prasugrel) but increased the risk of major bleeding (1.8% for clopidogrel versus 2.4% for prasugrel); these results suggest that prasugrel is a more potent $P2Y_{12}$ inhibitor than clopidogrel.

Protease-activated-receptor-1 inhibitors

The cleavage of protease-activated receptor 1 (PAR1) by thrombin activates platelets[5] (Fig. 2). PAR1 is a new target for antiplatelet therapy. Two PAR1 antagonists, E5555 and SCH 530348, are currently in phase II clinical trials[10].

$\alpha_{IIb}\beta_3$-Integrin inhibitors

The aggregation of platelets is a crucial step in the growth of a thrombus. Inhibitors of $\alpha_{IIb}\beta_3$-integrin are designed to reduce platelet aggregation by inhibiting the binding of activated platelets to fibrinogen and other ligands (Fig. 2). Intravenously delivered inhibitors of $\alpha_{IIb}\beta_3$-integrin, such as abciximab and eptifibatide, are used for the short-term treatment of patients with acute coronary syndromes who are undergoing percutaneous coronary intervention[52]. However, the use of oral $\alpha_{IIb}\beta_3$-integrin inhibitors is associated with increased mortality, possibly as a result of the longer-term use of these agents and/or their unwanted ability to act as partial agonists of $\alpha_{IIb}\beta_3$-integrin.

Late stent thrombosis

In patients with symptomatic coronary artery disease, insertion of a drug-eluting stent (delivering either sirolimus or paclitaxel) in the occluded coronary artery has become a popular treatment. So far, about 6 million people worldwide have received drug-eluting stents. The drugs are designed to prevent smooth muscle cell proliferation and intimal hyperplasia, which lead to re-occlusion of the vessel (that is, to restenosis), and randomized clinical trials have shown that drug-eluting stents reduce the rate of restenosis compared with bare metal stents[20]. Individuals receiving stents are initially treated with antiplatelet agents, such as clopidogrel (which inhibits the cell-surface receptor $P2Y_{12}$). However, recent reports have associated the use of drug-eluting stents with thrombosis after discontinuation of the antiplatelet therapy (a phenomenon known as late stent thrombosis)[21]. In a report examining the results of 14 randomized trials, the rate of late stent thrombosis was found to be fourfold-to-fivefold higher for drug-eluting stents than for bare metal stents, with thrombosis occurring with a median time of 15.5–18 months after stent deployment[22]. These results imply that patients with drug-eluting stents might benefit from prolonged antiplatelet therapy, although it is uncertain how long such therapy should continue. The triggering events for stent thrombosis are unclear but probably involve incomplete endothelialization of the stent surface[21]. Interestingly, the incidence of stent thrombosis was significantly lower in patients receiving a recently developed inhibitor of platelets, prasugrel, than in those receiving the more well-established drug in this class, clopidogrel (an incidence of 1.1% with prasugrel and 2.4% with clopidogrel), suggesting that prasugrel could be useful for this indication[23]. However, a better long-term option for reducing the incidence of late stent thrombosis will be the design of new types of stent able to deliver drugs that can reduce smooth muscle cell proliferation without interfering with endothelialization.

Venous thrombosis

Deep vein thrombosis and pulmonary embolism are collectively referred to as venous thromboembolism, which is the third leading cause of cardiovascular-associated death, after myocardial infarction and stroke. Deep vein thrombosis occurs most often in the large veins of the legs. Pulmonary embolism is a complication of deep vein thrombosis that can occur if part of the thrombus breaks away, travels to the lungs and lodges in a pulmonary artery, resulting in the disruption of blood flow. Thrombi that form in veins are rich in fibrin and trapped red blood cells and are referred to as red clots (as opposed to the platelet-rich thrombi that form in arteries, which are referred to as white clots). Deep vein thrombosis mainly occurs as a result of changes in the composition of the blood that promote thrombosis, changes that reduce or abolish blood flow, and/or changes to the vessel wall[24]. Both genetic and environmental factors can increase the risk of thromboembolism[25,26]. In inherited venous thrombotic disease, there can be increased activity or abundance of proteins that promote coagulation and/or decreased abundance of proteins that inhibit coagulation. For example, a specific point mutation (present in about 5% of Caucasians) in the gene encoding factor V results in a variant that is resistant to inactivation by the anticoagulant protease activated protein C and therefore leads to increased clotting[27]. Acquired risk factors for venous thromboembolism include cancer, obesity and major surgery[25,26]. Increased amounts of circulating tissue factor, which sits at the apex of the coagulation cascade (Fig. 3), might also trigger venous thrombosis[28–30].

Anticoagulants are used to treat a wide variety of conditions that involve arterial or venous thrombosis, including prevention of venous thromboembolism and long-term prevention of ischaemic stroke in patients with atrial fibrillation. The two main classes of anticoagulant drug are vitamin K antagonists and heparins, which target multiple proteases in the coagulation cascade (Fig. 3 and Box 2). As is the case for antiplatelet

Box 2 | Anticoagulant therapy

Anticoagulant drugs reduce the activity of various proteases in the coagulation cascade (Fig. 3) by directly inhibiting them, by inhibiting their post-translational modification or by increasing the activity of an anticoagulant. The advantages and disadvantages of the main types of anticoagulant are described below.

Vitamin K antagonists

Vitamin K antagonists are used for long-term anticoagulant therapy. These inhibitors, introduced more than 50 years ago, are the only orally active anticoagulants in clinical use today. They function by inhibiting the enzyme vitamin K epoxide reductase, which uses vitamin K to modify several coagulation proteins (factor VII, factor IX, factor X and prothrombin) post-translationally. Warfarin is the most commonly prescribed vitamin K antagonist; about 1% of the US population is currently being treated with this drug. Despite careful monitoring, the incidence of major bleeding is about 1–3% of warfarin-treated patients per year[53]. The activity of warfarin is affected by diet and by genetic make-up: polymorphisms in the gene that encodes vitamin K epoxide reductase and in the cytochrome P450 gene *CYP2C9* account for up to 50% of the interindividual variability of warfarin dosing[54]. In August 2007, the US Food and Drug Administration announced a label change for warfarin, advising that pharmacogenetic tests for polymorphisms in these two genes could improve the accuracy of dosing.

Heparins

The anticoagulant properties of unfractionated heparin were first described in 1916. Since then, it has become evident that heparin binds to the protein antithrombin and markedly increases the ability of this protein to inhibit factor Xa and thrombin (Fig. 3). Unfractionated heparin is currently used for cardiovascular surgery and for the prevention of venous thromboembolism. Fractionated heparin, in the form of low-molecular-weight heparins, was introduced more than 15 years ago. These molecules also target both factor Xa and thrombin, but their administration results in a lower incidence of bleeding than does unfractionated heparin (1.4% for low-molecular-weight heparins versus 2.3% for unfractionated heparin)[55]. Synthetic pentasaccharides, such as fondaparinux and idraparinux, have been designed with a structure based on the antithrombin-binding sequence of heparin[56]. Owing to their small size, these drugs target factor Xa but not thrombin in an antithrombin-dependent manner (as they are too short to stabilize the interaction between antithrombin and thrombin). A complication of administering unfractionated heparin is the syndrome of heparin-induced thrombocytopenia, which is associated with high rates of both arterial thrombosis and venous thrombosis. More specifically, heparin administration can result in the generation of antibodies specific for heparin–platelet-factor-4 complexes; these antibodies can then activate platelets, generating thrombin and leading to thrombosis[57]. The incidence of heparin-induced thrombocytopenia is reduced when low-molecular-weight heparins are used, and thrombocytopenia is rarely observed when synthetic pentasaccharides are used.

Direct inhibitors of factor Xa and thrombin

Direct thrombin inhibitors, such as lepirudin and desirudin, are used for anticoagulant therapy and for the treatment of patients with heparin-induced thrombocytopenia. Several orally administered agents are in development, including: the thrombin inhibitor dabigatran, which is as effective as the low-molecular-weight heparin enoxaparin at reducing the risk of venous thromboembolism after hip-replacement surgery and has a similar safety profile[58]; and the factor-Xa inhibitor rivaroxaban, which has a favourable balance of efficacy and safety for preventing venous thromboembolism after major orthopaedic surgery[33,59]. Several other orally administered direct inhibitors of factor Xa are also in the pipeline[40]. Further studies are required to determine whether oral inhibitors of thrombin or factor Xa can replace the use of heparins and warfarin for both short-term and long-term anticoagulant therapy.

drugs, the main side effect of anticoagulant therapy is bleeding. Which targets are best for anticoagulant therapy and whether the anticoagulant drugs under development will have better therapeutic windows than the existing drugs are topics of intense debate[31,32]. Recent data from the RECORD 1 clinical trial show that the anticoagulant rivaroxaban holds promise[33]. Rivaroxaban is an orally available inhibitor of activated factor X (factor Xa, a component of the coagulation cascade), and it reduced the incidence of venous thromboembolic events in patients undergoing total hip replacement — from 3.7% in those administered a low-molecular-weight heparin (enoxaparin) to 1.1% (ref. 33). This translates to a 70% reduction in risk without an increase in bleeding.

When targeting factors in the coagulation cascade, it is important to consider that the sequential activation of factors by proteolytic cleavage results in an amplification of each step. Therefore, a drug that targets an upstream component of the cascade, such as tissue factor, might be more potent than one that targets a downstream component, such as thrombin. However, the tissue factor and factor VIIa complex, which initiates the coagulation cascade, is essential for haemostasis, and inhibition of this complex can lead to considerable bleeding[34]. Indeed, gene-knockout experiments in mice have shown that tissue factor, as well as factor VII, factor X and prothrombin, are essential for haemostasis and for life[35].

It is also important to consider that the coagulation cascade can be subdivided into three pathways (Fig. 3): the extrinsic pathway (tissue factor and factor VIIa), which is the primary activator of the cascade; the intrinsic pathway (factor XIIa, factor XIa, factor IXa and factor VIIIa), which amplifies the cascade; and the common pathway (factor Xa, factor Va and thrombin), which generates thrombin and fibrin. In contrast to the critical nature of the extrinsic pathway, mice can survive without components of the intrinsic pathway[35]. Humans deficient in factor VIII, factor IX or factor XI have mild haemostatic defects, whereas those deficient in factor XII have normal haemostasis[36]. Intrinsic-pathway components might therefore be usefully targeted for therapy. Factor XIIa is of particular interest in this regard. A recent study with factor-XII-deficient mice confirmed that factor XIIa is not required for haemostasis; however, it was shown to be important for thrombosis and thus seems an inviting target for antithrombotic therapy[37]. Factor IXa, part of the intrinsic pathway, has also been proposed as a target[38]. Despite the possibility that the risk of bleeding is lower after inhibition of components of the intrinsic pathway than of the common coagulation pathway, most pharmaceutical companies have chosen to focus on inhibition of factor Xa and thrombin[39,40] (Fig. 3). This might be because inhibition of the intrinsic pathway is expected to have less impact on ongoing thrombosis than would inhibition of the downstream proteases.

An important concern about antithrombotic drugs is how to reverse their effects in the event of bleeding. A new approach that addresses this concern uses aptamers, which are composed of modified oligonucleotides. The first aptamer developed as an anticoagulant was targeted to thrombin and was shown to inhibit the activity of clot-bound thrombin and to reduce the formation of platelet-rich arterial thrombi[41]. More recently, an RNA aptamer that inhibits factor IXa has been developed[42]. By elegant design, an 'antidote' oligonucleotide was also developed, to reverse the anticoagulant activity of the inhibitory aptamer rapidly in the event of bleeding[43]. The factor-IXa aptamer–antidote pair was well tolerated in a phase Ia clinical trial with healthy volunteers[44]. In another approach using oligonucleotides, antisense therapy has been used to block not the activity of the target but its production (in this case, targeting prothrombin)[45].

Conclusions

The 'holy grail' for antithrombotic therapy — a drug that will prevent coagulation without promoting bleeding — has yet to be found. However, molecules that contribute to thrombosis continue to be identified, and these could be new targets for the next generation of antithrombotic therapy. More immediately, recent studies of new antiplatelet drugs (such as prasugrel) and new anticoagulant drugs (such as rivaroxaban) suggest

that more options will soon be available for the treatment of thrombosis. Furthermore, the ability to identify patients with an increased risk of thrombosis by measuring the concentrations of circulating factors, such as tissue factor, might allow more effective use of prophylaxis. Another exciting new approach is combination therapy, administering both antiplatelet drugs and anticoagulant drugs, which might prove more effective than using a single class of drug. Finally, personalized medicine is on the horizon and should allow customized dosing of antithrombotic drugs rather than the current 'one dose fits all' strategy. ■

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Tackling heart failure in the twenty-first century

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Heart failure, or congestive heart failure, is a condition in which the heart cannot supply the body's tissues with enough blood. The result is a cascade of changes that lead to severe fatigue, breathlessness and, ultimately, death. In the past quarter century, much progress has been made in understanding the molecular and cellular processes that contribute to heart failure, leading to the development of effective therapies. Despite this, chronic heart failure remains a major cause of illness and death. And because the condition becomes more common with increasing age, the number of affected individuals is rising with the rapidly ageing global population. New treatments that target disease mechanisms at the cellular and whole-organ level are needed to halt and reverse the devastating consequences of this disease.

> *Every day it grew worse, attacking him no longer at intervals, but relentlessly, with no interruption. He was unable to lie on either side, so weak that every breath involved great effort … his condition was serious, for never for one moment could he breathe freely. He was forced to sit upright in order to breathe at all; if by chance he did lie on his back or side, the suffocation was awful: to breathe in or exhale even a tiny stream of air became impossible.*
>
> A twelfth-century description of heart failure, from *The Alexiad*, by Anna Comnena (a biography of the Byzantine emperor Alexius I Comnenus)[1]

Heart failure, termed dropsy for centuries, was long considered an incurable disease with little hope of recovery. By the late twentieth century, insight into the role of haemodynamics and neurohormones in the disease, together with the development of effective treatments, had transformed heart failure into a chronic disease. However, the prevalence of heart failure worldwide continues to increase, and it has high rates of morbidity and mortality, imposing enormous human, social and economic costs[2]. Individuals with heart failure usually develop symptoms gradually, and they become increasingly less active and experience more frequent episodes of acute heart failure (known as decompensation). Treatment of the acute condition focuses on haemodynamics and has changed little in the past decade, whereas new research has largely targeted the chronic disorder. Therefore, in this article, we focus on chronic heart failure, and we highlight recent advances in understanding disease mechanisms and in developing new types of therapy, including direct targeting of intracellular proteins, delivering genes to repair enzyme abnormalities, replacing cell populations and implanting microprocessors. Many of these advances stem from insights into the intracellular signalling pathways that control cardiac hypertrophy and dilation, myocardial energetics, cellular calcium cycling and the contractile machinery itself, and we discuss these in detail.

The heart-failure syndrome

Heart failure develops when the heart can no longer provide adequate blood flow and/or pressure to meet the body's demands. This failure triggers countermeasures, including the retention of salt and water by the kidneys, the stimulation of the body's organs by neurohormones, and the activation of intracellular signalling cascades in the heart and vasculature that alter cellular and organ morphology and function. Such 'compensatory' responses can initially offset reduced cardiac performance, but they become key 'co-conspirators' in the disease process, ultimately increasing the likelihood of organ failure and worsening clinical prognosis. Although individuals with heart failure have some symptoms in common, including fatigue, shortness of breath and fluid retention, the clinical presentation of heart dysfunction is heterogeneous. About half of all individuals have contractile failure and a dilated heart (that is, systolic heart failure), and the other half seem to have normal contraction and a non-dilated, but often hypertrophied, heart (that is, heart failure with a preserved ejection fraction (HFpEF), which has also been termed diastolic heart failure). HFpEF is increasing in prevalence worldwide but remains understudied.

In recent decades, therapy for heart failure has undergone several large shifts, moving from a focus on haemodynamics to a focus on the targeting of specific disease mechanisms. One such shift is that neurohormonal stimulation is no longer viewed as always having beneficial effects but as often worsening heart failure, and it is therefore a process that should be blocked. For example, arterial vasodilators have long been known to have therapeutic effects through reducing the workload of the heart; however, more recently, inhibitors of the renin–angiotensin–aldosterone system were found to be more effective than arterial vasodilators, even though they have similar effects on workload. An even more remarkable example is the use of β-blockers, which antagonize the activation of β-adrenergic receptors (β-ARs) on cardiomyocytes by the sympathetic nervous system. Although β-blockers were long considered to be contraindicated for heart failure, they were ultimately shown to improve outcome and are now established therapeutics. A second shift has been to avoid treatments designed to stimulate muscle in the weakened heart. Although such therapy helped in the short term, it was generally found to be detrimental when carried out long term[3]. A third shift is that a 'bionic' era is now dawning, in which implantable devices controlled by microprocessors can deliver therapy, monitor disease and rescue a patient from sudden cardiac death. Since the turn of the twenty-first

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century, these devices have had the most impact of any new treatment for heart failure.

Blocking neurohormones

After heart function declines, the body compensates by increasing stimulation by the sympathetic nervous system (through adrenaline and noradrenaline) and neurohormonal pathways (for example, through angiotensin II, endothelin and natriuretic peptides). These changes increase the rate and the intensity of heart contraction and the amount of fluid retention and thereby help to maintain cardiac output. In humans with heart failure, the abundance of neurohormones and neurotransmitters is correlated with heart function, and studies in animal models have provided insight into the intracellular signalling pathways triggered by these stimuli and their effects on the heart. Sustained stimulation is toxic and portends a poor prognosis[4]. For example, mice that are genetically engineered to overproduce β_1-AR or stimulatory G proteins (G_s, G_q or G_{11}, which couple to different receptor types) develop heart failure, whereas those deficient in components of these signalling pathways are protected[5,6]. However, modulating β-AR signalling pathways in other ways to increase particular effects of adrenergic stimulation — such as blunting the activity of G-protein-coupled receptor kinase 2 (ref. 7) or upregulating the expression of the gene encoding adenylyl cyclase 6 (ref. 8) — can benefit the failing heart. Thus, the precise nature of the stimulation is important for determining its effect on heart function.

Different stresses on the heart stimulate different signalling pathways. For example, in mice both the physiological stress of exercise and the pathological stress of pressure overload (mimicking chronic high blood pressure) stimulate processes that lead to cardiac hypertrophy, but these stresses couple to different signalling pathways. Physiological stresses trigger signalling through the serine/threonine kinase Akt, the transcription factor STAT3 (signal transducer and activator of transcription 3) and the cytokine receptor gp130 (also known as interleukin-6 signal transducer). By contrast, pathological stresses trigger signalling through various molecules, including $G_{q/11}$-protein-coupled receptors, calcium (Ca^{2+})/calmodulin-dependent kinase II (CAMKII), mitogen-activated protein kinases (MAPKs), the protein phosphatase calcineurin, and the transcription factors myocyte enhancer factor 2 (MEF2) and nuclear factor of activated T cells (NFAT)[9]. It is tempting to speculate that the type of signalling triggered depends on whether the stress is intermittent (as with exercise) or chronic (as with pressure overload). However, this does not seem to be the case, because intermittent pressure overload induces a pathological 'molecular footprint' that differs from that induced by exercise[10].

Therapies found to improve survival and symptoms in individuals with heart failure include blockade of β-ARs, inhibition of angiotensin-converting enzyme, blockade of the angiotensin II receptor AT1, and inhibition of aldosterone synthesis[11–13]. Long-term β-AR blockade can reverse the downregulation of β-ARs, increase signalling through inhibitory G proteins and G-protein-coupled receptor kinase 2 (see ref. 14 for a review) and ameliorate abnormalities in the production of excitation–contraction-coupling proteins[15]. AR blockers vary in the β-AR subtype they target (β_1 or β_2) and whether they target α_1-AR, and these variations might underlie the differing effects of these drugs. For example, in contrast to what might have been expected, an increase in β_2-coupled stimulation might protect against cardiomyocyte death (by apoptosis or necrosis)[16]. In addition, through signalling involving β-arrestin, β_1-AR can transactivate the epidermal growth factor receptor (EGFR) signalling pathway[17], which is cardioprotective; this finding suggests that β-blockers designed to stimulate this pathway would have increased therapeutic benefit.

Endothelin and various pro-inflammatory cytokines contribute to the pathophysiology of heart failure; however, the pharmacological blockade of these molecules has yielded disappointing results, perhaps owing to the complexity of their signalling effects. For example, endothelin interacts with two receptor subtypes (ET_A and ET_B) that have opposing effects. Recently developed endothelin blockers, such as ambrisentan, target only ET_A, so they might be more effective than previous candidates. Targeted blockade of the pro-inflammatory cytokine tumour-necrosis factor proved disappointing and even led to a worse clinical outcome[18], and the efficacy of broader anticytokine approaches remains uncertain. Modulating the natriuretic peptides — atrial natriuretic peptide (ANP) and brain natriuretic peptide (BNP), which are thought to be cardioprotective — seems more promising. The concentrations of endogenous circulating (plasma) ANP and BNP are often increased in heart failure — indeed, BNP concentrations are used as a marker of disease severity — but their effects on heart failure might be blunted as a result of depressed responsiveness of their target organs, including the heart. Exogenous infusion of BNP has been used clinically, and a large clinical trial assessing its effects on mortality is now in progress. Furthermore, it might be possible to enhance responsiveness to natriuretic peptides by suppressing the hydrolysis of cyclic GMP, through administering phosphodiesterase 5 (PDE5) antagonists such as sildenafil[19].

Pharmacogenomic profiling

From advances in understanding the genetic basis of human disease, it is clear that inherited cardiomyopathy can result from a single mutation in one of many dozens of genes, including those encoding proteins that make up the contractile machinery of cardiomyocytes, proteins that regulate Ca^{2+} cycling or energy metabolism, and proteins that regulate transcription[20]. In most patients, however, cardiomyopathy results not from mutation in one of these genes but from interactions between the proteins encoded by a larger number of modifier genes, together with effects from the environment[21]. Genetic differences also affect how patients respond to drugs, and attempts are now underway to uncover the genes that are responsible, which could, eventually, allow individualized therapy. Ethnicity might contribute to these variations: for example, African Americans benefit more than Caucasians from combined treatment with hydralazine and isosorbide[22], although the specific genetic differences responsible remain unclear.

A better-defined example of how genetic differences affect responses to drugs is the discovery that polymorphisms in ARs can result in different responses to sympathetic stimulation and AR-antagonist administration. Patients with heart failure who have the β_2-AR polymorphism in which isoleucine is present at position 164 have an almost fivefold higher risk of cardiac-associated death or need for heart transplantation[23], and polymorphisms in both β_1-AR and β_2-AR are linked to lower exercise tolerance. In addition, African Americans with both a polymorphism in β_1-AR that increases its activation and an polymorphism in α_1-AR that decreases presynaptic noradrenaline uptake have a higher risk of heart failure[24,25]. Another example is that a polymorphism resulting in the presence of either arginine or glycine at position 389 of β_1-AR affects responses to β-blocker in patients with heart failure: those homozygous for the arginine-encoding variant have lower mortality and fewer hospitalizations, whereas those with the glycine-encoding variant have little response to therapy[26,27]. Polymorphisms in the promoter of the gene encoding $G\alpha_q$ that adversely affect survival in African Americans with heart failure have also been identified[28]. To uncover further how genetic variation can affect the response of patients to therapy, analysis of genotype–phenotype associations in large populations is underway (for example, using data from the Framingham Heart Study; http://www.ncbi.nlm.nih.gov/projects/gap/cgi-bin/study.cgi?id=phs000007).

Untangling the molecular web

The failing heart is heavy, with an increased muscle-wall mass as a result of cardiomyocyte hypertrophy. This generally occurs together with either chamber enlargement and weakened contraction (that is, systolic cardiomyopathy) or wall thickening and preserved contraction (that is, HFpEF), the latter of which are seen in hypertensive heart disease. A broad range of molecular pathways are thought to be involved in the development of both types of cardiomyopathy (Fig. 1), and there is likely to be substantial overlap. Typically, cell-surface receptors are activated by the binding of a ligand or by a mechanical stimulus, and this leads to the activation of stress-response protein kinases and phosphatases,

such as cyclic-AMP-dependent protein kinase (PKA), protein kinase C (PKC), protein kinase D (PKD), MAPKs, CAMKII and calcineurin (Fig. 1). These enzymes, in turn, activate transcription factors, which target multiple genes, and the result is a change in the cellular structure, size, shape and molecular regulation of the heart, often collectively referred to as cardiac remodelling. Early efforts aimed at finding a common pathway that could be manipulated to rescue pathological cardiac remodelling were frustrated by the identification of complex parallel signalling cascades. Nonetheless, there seem to be crucial signalling 'nodes' (points at which several pathways converge) — for example, glycogen synthase kinase 3β (GSK3β) and histone deacetylases (HDACs) — and remodelling signals that can be modulated by administering small molecules have been identified (see ref. 29 for a review).

An early proposed target for treatment of heart failure was calcineurin, a serine/threonine phosphatase that dephosphorylates NFAT molecules, allowing them to translocate to the nucleus, where they activate a hypertrophic genetic programme. This pathway is crucial for cardiac hypertrophy and remodelling in animal models[30]. Calcineurin activity is inhibited by cyclosporin A and FK506, immunosuppressants used in organ transplantation, but the side effects of these agents limit their usefulness for treating heart disease. Subsequently, endogenous inhibitors of calcineurin activity were discovered — including AKAP1 (PKA anchor protein 1), atrogin 1 (also known as FBXO32) and regulator of calcineurin 1 (RCAN1; formerly known as MCIP1) — so targeting these might be a more selective and effective approach to inhibiting the calcineurin–NFAT pathway. Other mediators of hypertrophic cardiac growth are the δ-subunit of CAMKII, which might have a role in heart-failure progression and arrhythmia[31]; PKC-α, which can suppress contractility by increasing the dephosphorylation of phospholamban (a protein that modulates cytosolic Ca^{2+} concentrations)[32]; and MAPKs, in particular p38 MAPK, which seems to exacerbate fibrosis and induce contractile dysfunction[33].

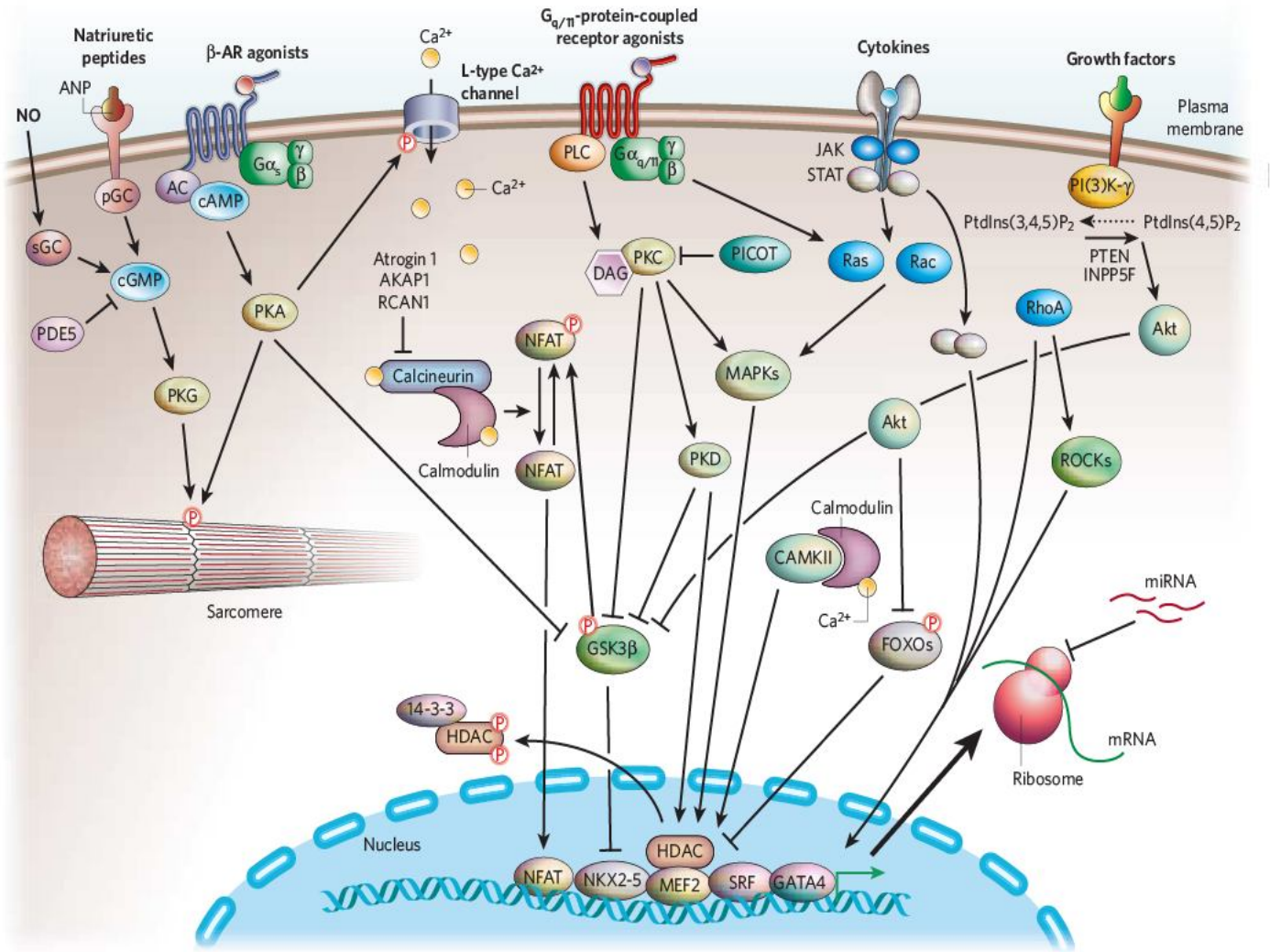

Figure 1 | Cardiomyocyte signalling pathways involved in the pathophysiology of heart failure. Stress stimuli are transduced by many intracellular signalling pathways and ultimately result in changes in cardiomyocyte function and growth. A schema of these signalling pathways is shown; for simplicity, only some of the known interactions and feedback loops are depicted. Stress stimuli include nitric oxide, neurohormones (such as natriuretic peptides and angiotensin II, the latter of which binds to G_q- or G_{11}-protein-coupled receptors), neurotransmitters (such as catecholamines, which bind to β-adrenergic receptors (β-ARs)), cytokines and growth factors. After cell-surface receptors bind to these ligands, the signal is transmitted to protein kinases, which in turn activate signalling nodes (where many pathways converge). These nodes include calcium (Ca^{2+})/calmodulin-dependent kinase II (CAMKII), Akt, glycogen synthase kinase 3β (GSK3β) and cyclic GMP (cGMP)-dependent protein kinase (PKG). These pathways are involved in physiological responses; however, in the failing heart, there are more stress stimuli, thereby amplifying these pathways and generating imbalances among them. AC, adenylyl cyclase; AKAP1, PKA anchor protein 1; ANP, atrial natriuretic peptide; cAMP, cyclic AMP; DAG, diacylglycerol; FOXO, forkhead-box O proteins; HDAC, histone deacetylase; INPP5F, inositol polyphosphate-5-phosphatase F; $InsP_3$, inositol-1,4,5-trisphosphate; JAK, Janus kinase; MAPK, mitogen-activated protein kinase; MEF2, myocyte enhancer factor 2; mRNA, messenger RNA; miRNA, microRNA; NFAT, nuclear factor of activated T cells; NKX2-5, NK2 transcription factor related, locus 5; NO, nitric oxide; pGC, particulate guanylyl cyclase; PICOT, PKC-interacting cousin of thioredoxin; PI(3)K-γ, phosphatidylinositol 3-OH-kinase-γ; PKA, cAMP-dependent protein kinase; PKC, protein kinase C; PDE5, phosphodiesterase 5; PKD, protein kinase D; PLC, phospholipase C; $PtdIns(4,5)P_2$, phosphatidylinositol-4,5-bisphosphate; PTEN, phosphatase and tensin homologue; RCAN1, regulator of calcineurin 1; ROCK, Rho-associated, coiled-coil-containing protein kinase; sGC, soluble guanylyl cyclase; SRF, serum response factor; STAT, signal transducer and activator of transcription.

In addition to these activators of hypertrophy, there are also negative modulators of hypertrophy, the inactivation of which probably has an important role in heart failure. An example is GSK3β, a serine/threonine kinase that regulates cellular development, cycling, metabolism, apoptosis and gene transcription[34] (Fig. 1). Modulation of GSK3β activity regulates hypertrophic growth: GSK3β is normally active, thereby inhibiting hypertrophy, but phosphorylation of GSK3β by Akt or PKA suppresses its activity, thereby releasing hypertrophic pathways from this negative control. Mice that produce a constitutively active form of GSK3β fail to show hypertrophy after pressure overload[35], but the implications of this antihypertrophic effect for treatment of heart failure remain debatable. A recent study found that mice that produce a dominant-negative form of GSK3β respond to pressure overload by developing a physiological form of hypertrophy that has beneficial effects on heart function, whereas mice that produce gain-of-function forms of GSK3β have a more impaired heart function than that of wild-type mice[36]. GSK3β is also activated indirectly by the phosphatases PTEN (phosphatase and tensin homologue) and INPP5F (inositol polyphosphate-5-phosphatase F), both of which decrease concentrations of the phosphoinositide phosphatidylinositol-3,4,5-trisphosphate ($PtdIns(3,4,5)P_3$), thereby blunting signalling by the phosphatidylinositol 3-OH-kinase (PI(3)K)–Akt pathway[37,38]. In contrast to GSK3β, GSK3α has antihypertrophic effects when phosphorylated and does not prevent apoptosis or necrosis[39]. Similarly to GSK3β, the FOXO subfamily of forkhead-box transcription factors are also negative growth modulators that are inactivated on phosphorylation[40] (Fig. 1). Modulation of FOXO- and GSK3β-mediated signalling might prove useful for treating individuals with failing hearts.

Cyclic GMP and cGMP-dependent protein kinase (PKG) are also endogenous negative modulators of stress-response signalling. The generation of cGMP occurs when natriuretic peptide binds to receptors coupled to guanylyl cyclase or when soluble guanylyl cyclase is directly activated by nitric oxide (Fig. 1). Multiple functions are known for cGMP, including activating PKG and controlling the activities of various phosphodiesterases (for example, PDE2, PDE3 and PDE5), which in turn control cAMP and cGMP breakdown. Well-known targets of PKG include Ca^{2+} channels in vascular smooth muscle cells, the target subunit of myosin phosphatase, RGS2 (which negatively regulates G-protein-coupled receptor signalling) and IRAG (also known as MRVI1, which regulates inositol-1,4,5-trisphosphate ($InsP_3$)-dependent Ca^{2+} signalling). In cardiomyocytes in particular, the targets of PKG include troponin I (which is a component of the contractile machinery), phospholamban and possibly RGS2 (ref. 41). PKG can be activated by blocking the hydrolysis of cGMP; for example, with the PDE5 inhibitor sildenafil. PDE5 inhibitors are used clinically to treat erectile dysfunction and pulmonary hypertension, but they have been shown to have additional benefits in a variety of animal models: they protect against ischaemia–reperfusion injury, mitigate myocardial apoptosis stimulated by the chemotherapeutic agent anthracycline, suppress acute and chronic β-adrenergic stimulation, and blunt and reverse pressure-overload-induced cardiac hypertrophy[42,43]. On the basis of these findings[42,43], which were mainly from animal studies, a trial sponsored by the National Institutes of Health trial is underway to test the therapeutic value of PDE5 inhibitors for treating HFpEF.

Various transcription factors function as more distal effectors of cardiac remodelling in response to stress signals: MEF2, GATA-binding protein 4 (GATA4), NFAT and several HDACs all seem to be important for such remodelling[9] (Fig. 1). HDACs promote the condensation of chromatin, thereby preventing transcription factors from accessing DNA. Class II HDACs (namely HDAC4, HDAC5, HDAC7A and HDAC9) negatively regulate pathological, but not physiological, hypertrophy in a Ca^{2+}-dependent manner[44]. The phosphorylation of class II HDACs by any of several stress-activated kinases (for example, PKD or CAMKII) results in binding of the HDAC to the chaperone protein 14-3-3, dissociation of the HDAC from the transcriptional machinery (resulting in derepression of MEF2 activity) and export of the HDAC from the nucleus[45,46]. Conversely, a class I HDAC, HDAC2, promotes hypertrophy by repressing expression of *Inpp5f*, resulting in inactivation of GSK3β through effects on PI(3)K–Akt signalling[38]. Therefore, agents that inhibit HDAC2 or prevent class II HDAC phosphorylation might be able to control stress-activated growth. The transcription factor GATA4 is activated by MAPKs (extracellular-signal-regulated kinase 1 (ERK1), ERK2 or p38 MAPK) and by GSK3β (coupled to Akt inactivation) in cardiomyocytes from failing or hypertrophied hearts, but activated GATA4 seems to have mainly a beneficial role, allowing adaptive hypertrophic remodelling and cytoprotection. In support of this finding, deletion of the gene encoding GATA4 in the adult heart results in cardiac dysfunction, chamber dilation and increased apoptosis[47].

An exciting recent discovery has been the identification of a class of small RNAs, termed microRNAs (miRNAs), that negatively regulate gene transcription or translation and are involved in many physiological and pathological processes. Among the many miRNAs, it has been reported that miR-21, miR-133 and miR-208 are key regulators of cardiac hypertrophy, with miR-133 having antihypertrophic effects and miR-208 having prohypertrophic effects[48,49]. In addition, an miRNA (miR-1) has been implicated in promoting arrhythmogenesis in the border zone of an infarct (where a heart attack has occurred), by reducing the amount of connexin 43 (a gap-junction protein) and potassium channel proteins produced, thereby possibly slowing electrical conduction in the heart muscle[50]. Blocking the actions of miRNAs is an intriguing therapeutic opportunity, because these molecules can be inhibited by intravenously delivering oligonucleotides known as antagomirs[51] or agents called sponges, which block miRNA activity more broadly[52]. This field is developing rapidly and could yield a new approach to heart-failure therapy.

Many of the signalling pathways discussed in this section trigger the generation of reactive oxygen species (ROS), a process that is increasingly recognized as an important contributor to depressed cardiac function and maladaptive remodelling[53]. There are several sources of ROS, including the NADPH-oxidase system (which can be activated by angiotensin II and other stimuli), xanthine oxidase, monoamine oxidases (which are important for catecholamine and serotonin catabolism), mitochondrial electron leak and nitric-oxide synthase 3 (NOS3; also known as eNOS). Because the site where ROS are generated in the cell depends on the stimulus, and because the effects of ROS are likely to depend on where they are generated, targeted inhibition of the mechanisms that generate ROS might be a more successful treatment than the administration of broad-acting antioxidants has been.

The role of NOS3 in generating ROS and contributing to heart dysfunction is worth highlighting, because NOS3 is usually considered to protect against oxidative cytotoxicity, abnormal growth, and fibrosis. In an oxidative environment, the normal electron transfer from the reductase domain to the oxygenase domain of NOS3 can be impaired ('uncoupled'), resulting in decreased synthesis of nitric oxide and increased synthesis of superoxide. Uncoupled NOS3 activity seems to contribute to the pathology of the hypertrophied and failing heart[54]. Administration of tetrahydrobiopterin, an obligate cofactor for NOS enzymes, might be able to counter this uncoupled activity[54], but this hypothesis needs to be tested in clinical trials.

Matters of life and death or replacement

In the failing heart, an imbalance between signalling pathways that promote cell survival and those that promote cell death (through apoptosis or necrosis) results in a decrease in the number of cardiomyocytes. Apoptosis can be initiated by multiple pathways — including those triggered by ROS, angiotensin II, sympathetic stimulation or cytokines — and a low, but significantly abnormal, rate of cardiomyocyte apoptosis seems to contribute to the phenotype of heart failure[55,56]. Prosurvival pathways that counter apoptosis mainly involve JAK (Janus kinase)–STAT signalling[57] and PI(3)K–Akt signalling (in the latter case associated with nuclear translocation of Akt[58]). Suppression of cardiomyocyte apoptosis by increasing the activity of Akt or by blocking the activation of caspases (a family of calcium-dependent cysteine proteases central to apoptosis) might hold promise for therapy[55]. Necrosis also contributes

to heart failure, and it has recently been linked to mitochondrial damage stimulated by increasing the cytosolic Ca^{2+} concentration in myocytes. A recent report found that augmenting Ca^{2+} entry through the L-type Ca^{2+} channel triggered the opening of mitochondrial permeability transition pores by activating cyclophilin D (also known as PPID)[59], thereby inducing necrosis. Inhibiting cyclophilin D was a potent way to suppress this loss of myocytes.

Another mechanism that could contribute to cardiomyocyte loss is a highly conserved process termed autophagy, which can be induced by cellular starvation and is characterized by the recycling of proteins within organelles. Autophagy is observed in hypertrophied and failing hearts, but whether it has an adaptive or maladaptive role remains a matter of debate. Cardiac remodelling induced by pressure overload in mice has been shown to be ameliorated by silencing of the gene encoding beclin 1 — a protein required for autophagosome formation — and exacerbated by overproduction of beclin 1 (ref. 60). By contrast, mice lacking the protein ATG5 — which is also required for autophagy — developed dilated cardiomyopathy, and mice with a cardiac-specific deletion of the gene encoding ATG5 developed more hypertrophy in response to pressure overload than wild-type mice[61].

Given that cardiomyocytes undergo apoptosis and necrosis in individuals with heart failure, efforts to promote cardiac regeneration have taken centre stage (see page 937). Most studies have used infarcted hearts, trying to restore cardiomyocytes locally near the scar, but it is more difficult to consider applying this strategy to a heart with a global dysfunction. However, recent findings indicate that the effects of cell-based therapy might derive not from the replacement of cardiomyocytes but from the paracrine effects of the injected cells on endogenous cells[62]. Thus, a cocktail of factors that can provide such paracrine effects might prove beneficial.

Ca^{2+} signalling

Ca^{2+} is the central regulator of excitation–contraction coupling, which drives cyclical muscle contraction. Excitation–contraction coupling involves modulation of the cytosolic Ca^{2+} concentration: Ca^{2+} is released from the sarcoplasmic reticulum through the ryanodine channel (also known as the ryanodine receptor, RYR2), and this is followed by re-uptake of Ca^{2+} into the sarcoplasmic reticulum by a Ca^{2+}-uptake pump (SERCA2A) and removal of Ca^{2+} from the cell by the Na^+/Ca^{2+} exchanger (Fig. 2). The activity of SERCA2A is regulated by phospholamban. In its unphosphorylated state, phospholamban inhibits SERCA2A activity, but on phosphorylation (typically by PKA), this inhibition is released, thereby increasing Ca^{2+} uptake. In the failing heart, Ca^{2+} uptake into the sarcoplasmic reticulum is impaired, and Ca^{2+} concentrations in the sarcoplasmic reticulum are therefore decreased; these defects have been ascribed to a decline in SERCA2A production, reduced levels of phospholamban phosphorylation, and depletion of sarcoplasmic-reticulum Ca^{2+} through leaky RYR2 channels (see ref. 63 for a review).

At present, several of these processes are being targeted for therapy. Transfer of the gene encoding SERCA2A, deletion of the gene encoding phospholamban or production of a constitutively phosphorylated form of phospholamban improves heart function and remodelling in various animal models of heart failure[64], and clinical trials testing *SERCA2A* gene transfer are underway. Another therapeutic approach that is under study was inspired by findings that phospholamban is potently inhibited by the serine/threonine phosphatase PP1, which in turn is inhibited by the phosphatase inhibitor I-1 (also known as PPP1R1A), after I-1 has been phosphorylated by PKA (Fig. 2). Mice overproducing an activated form of I-1 are protected from heart failure[65], and transfer of a gene encoding an activated form of I-1 might soon be tested in clinical trials.

Repairing defects in Ca^{2+} release from the sarcoplasmic reticulum is also being explored as a potential therapy. The phosphorylation of RYR2 by PKA leads to dissociation of the protein calstabin 2 (also known as FKBP1B) (Fig. 2), and this dissociation is proposed to increase leakage of Ca^{2+} from the sarcoplasmic reticulum[66] and thus arrhythmogenicity. Blockade of β-ARs can help to prevent this Ca^{2+} leakage[67]. Other research groups have proposed that phosphorylation of RYR2 by CAMKII — rather than by PKA — is important for Ca^{2+} leakage through RYR2 (ref. 68). Administration of small-molecule inhibitors of CAMKII or stabilizers of the calstabin-2–RYR2 complex[69] could be new approaches to ameliorating arrhythmia and maladaptive heart remodelling.

Ca^{2+} function seems to be compartmentalized into microdomains in cardiomyocytes, because the marked swings in Ca^{2+} concentrations that occur in a cell as it contracts and relaxes do not themselves trigger Ca^{2+}-dependent signalling pathways. Some compartmentalization is at the plasma membrane; for example, localized Ca^{2+} entry through L-type Ca^{2+} channels in caveolae regulates β_2-AR signalling[70]. Other Ca^{2+}-stimulated proteins such as calsarcin 1 (also known as MYOZ2) localize to the Z-disk (a component of the sarcomere), where they might couple mechanical forces with Ca^{2+}-dependent enzymes, such as calcineurin, and subsequent transcriptional regulation[71]. A perinuclear Ca^{2+} pool under the control of the $InsP_3$ receptor regulates nuclear transport of HDACs (Fig. 2); this regulation is partly mediated by CAMKII and is independent of total cellular Ca^{2+} concentrations[72]. Another important pool of Ca^{2+} is generated by the influx of Ca^{2+} into the cell through channels known as transient receptor potential channels (TRPCs) (Fig. 2). These channels are referred to as store operated because the opening of these channels is coupled to a decline in $InsP_3$-regulated intracellular Ca^{2+} stores. TRPC activation might have an important role in the signalling pathways that lead to hypertrophy, with recent studies finding that TRPC1, TRPC3 and TRPC6 are involved in this process[73–75]. These studies have yielded discrepant results regarding which TRPC molecules are most important in pressure overload, and more work is needed to clarify the situation. TRPC6 is intriguing in that it might have a role both in exacerbating cardiac remodelling in response to stress such as pressure overload[75] and in renal glomerular dysfunction and proteinuria[76]. Thus, TRPC6 could be a therapeutic target for ameliorating both heart dysfunction and kidney dysfunction in patients with heart failure.

The starving heart

The heart has a high and constant workload, and cardiac energy supply and metabolism are tightly regulated. This regulation becomes compromised in the failing heart, which can lead to a state of inefficiency and energy starvation. Unlike other organs, the heart has a limited capacity for storing fuel, so substrates need to be produced efficiently and quickly, mainly from circulating free fatty acids and, to a lesser degree, from glucose. In the failing heart, the synthesis of ATP is compromised, partly as a result of mitochondrial dysfunction and, probably, altered substrate utilization (that is, increased catabolism of glucose).

The PGC1 family of transcriptional coactivators comprises important regulators of mitochondrial function, with PGC1α (peroxisome-proliferator-activated receptor-γ (PPAR-γ) coactivator 1α) being the best-studied family member in the heart. PGC1α functions to increase the level of oxidative phosphorylation to meet the energy demands of cardiac growth in response to physiological stimuli — that is, during development or in response to exercise — for which the heart uses mainly free fatty acids[77] (Fig. 3a). Mechanistically, PGC1α induces mitochondrial biogenesis by increasing the production of transcription factors — including nuclear respiratory factor 1 (NRF1) and NRF2 (which, in turn, regulate mitochondrial transcription factor A) and oestrogen-related receptor-α (ERR-α) — that coordinate the expression of genes encoding mitochondrial proteins. PGC1α also regulates the expression of genes encoding proteins involved in fatty-acid oxidation and respiration, through coactivation of PPAR-α and ERR-α[78]. The expression of the gene encoding PGC1α and of its downstream targets is downregulated in mouse models of pathological heart hypertrophy and failure, which is consistent with the depressed mitochondrial function seen in these models. Furthermore, PGC1α-deficient mice have an exacerbated heart-failure phenotype in response to pressure overload[79]. In the same study, hypertrophy induced in neonatal rat cardiomyocytes by $G_{q/11}$-protein-coupled receptor agonists leads to reduced expression of the gene encoding PGC1α and its gene targets, and cellular remodelling by these agonists is blunted by increasing PGC1α production[79].

Therefore, increasing PGC1α activity might be therapeutically useful for normalizing energy metabolism in the stressed heart.

Studies of non-ischaemic heart failure have found a shift in cardiac metabolism away from the normal preference for fatty acids and towards glucose[80]. Whether this shift results in differences in substrate oxidation and thus mitochondrial function and efficiency remains unclear. The situation seems to be better defined for ischaemic heart failure, in which glucose oxidation is initially favoured, leading to improved energy efficiency. By contrast, in the heart of individuals with type 1 diabetes, fatty-acid oxidation is favoured as a consequence of reduced glucose uptake and of PPAR-α activation by fatty-acid accumulation (see ref. 81 for a review). Because the oxygen cost of energy production is lower when using glucose than when using fatty acids, agents that can suppress fatty-acid uptake or oxidation (for example, oxfenicine, etomoxir and ranolazine) are being explored as treatments for heart failure associated with diabetes (see ref. 82 for a review).

Abnormalities in ATP storage are another aspect of dysregulated energy metabolism in failing hearts. Creatine kinase reversibly converts phosphocreatine and ADP to creatine and ATP when energy is needed rapidly; in this way, phosphocreatine provides a store of ATP (Fig. 3b). The ratio of the concentrations of phosphocreatine and ATP has been used as a measure of this energy balance, and in human heart failure there are abnormalities in this ratio and in ATP flux[83]. Changes in the ATP/AMP ratio are linked to metabolism by AMP kinase, which is a serine/threonine kinase that responds to decreased intracellular energy levels by stimulating fatty-acid oxidation, glucose uptake and glycolysis.

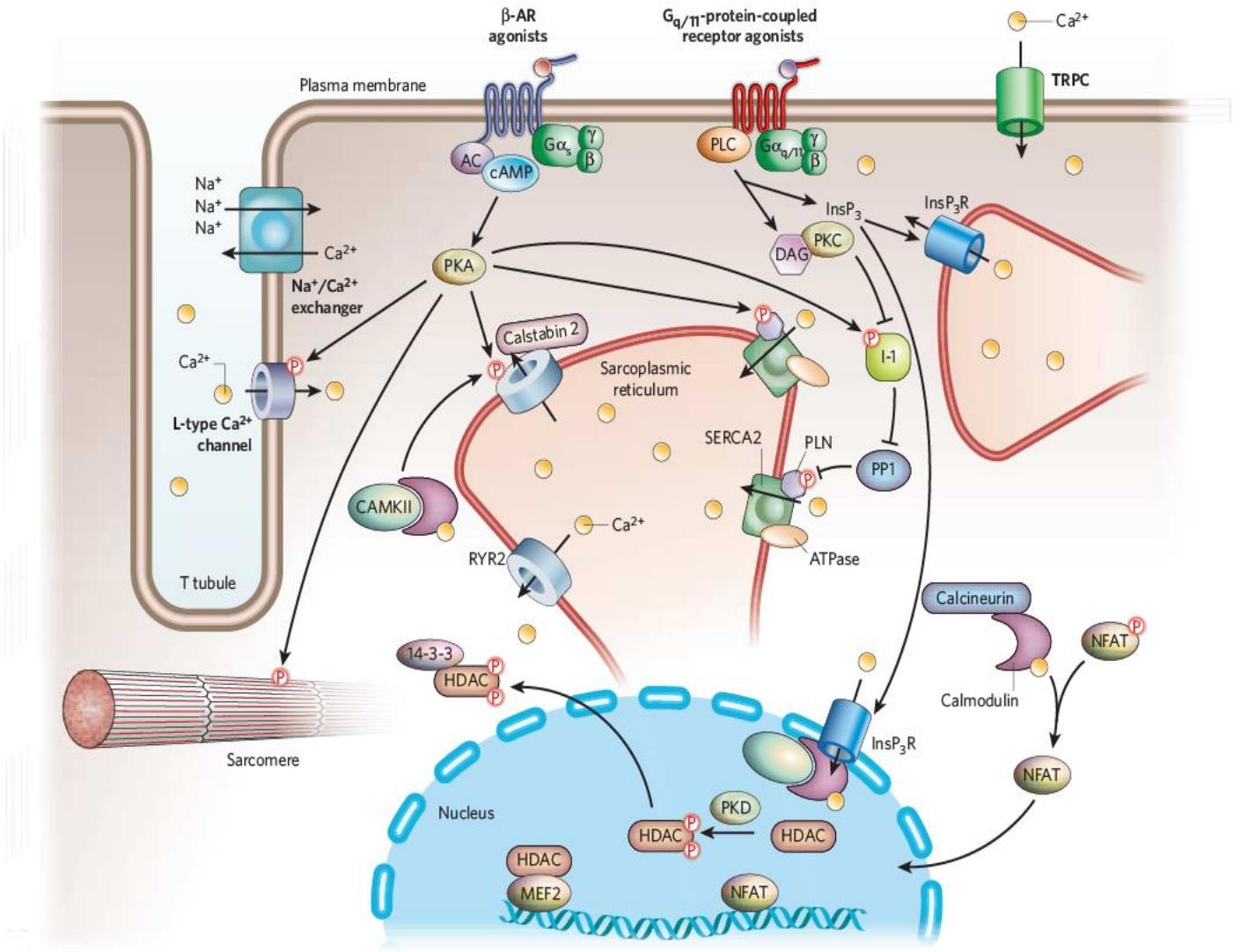

Figure 2 | Ca^{2+}-handling abnormalities in myocytes in the failing heart. The entry of Ca^{2+} to a cardiomyocyte through L-type Ca^{2+} channels stimulates the release of Ca^{2+} from the sarcoplasmic reticulum through RYR2, leading to activation of myofilaments (in the sarcomere). Resting Ca^{2+} concentrations are restored mainly through re-uptake of Ca^{2+} into the sarcoplasmic reticulum, by the Ca^{2+}-uptake pump SERCA2A, which is regulated by phospholamban (PLN). Ca^{2+} is also removed from the cell through the sodium (Na^+)/Ca^{2+} exchanger. The binding of β-adrenergic agonists to β-ARs results in the activation of PKA, which leads to the phosphorylation of the L-type Ca^{2+} channel, RYR2, PLN and sarcomere proteins (for example, troponin I, myosin-binding protein C and titin). This process increases both cellular contraction and relaxation, through the delivery of more Ca^{2+} to the myofilaments (increasing contraction) and the improved re-uptake of Ca^{2+} by the sarcoplasmic reticulum and desensitization of the myofilaments to Ca^{2+} (increasing relaxation). In addition to Ca^{2+} entry through L-type Ca^{2+} channels, Ca^{2+} release through RYR2 is also modulated by the interaction of RYR2 with calstabin 2. In the failing heart, there is depressed PKA activity, reduced Ca^{2+} re-uptake by the sarcoplasmic reticulum, increased Ca^{2+} extrusion through the Na^+/Ca^{2+} exchanger, and increased RYR2 phosphorylation and calstabin-2 dissociation. In addition, there is increased activation of $G_{q/11}$-protein-coupled receptor signalling, which in turn increases PKC-α activity. PKC-α then blocks activity of the phosphatase inhibitor I-1, thereby increasing the activation of the serine/threonine phosphatase PP1. This further reduces PLN phosphorylation and depresses both cellular contraction and relaxation, by preventing re-uptake of Ca^{2+} by the sarcoplasmic reticulum. Activation of $G_{q/11}$-protein-coupled receptors also increases the amount of $InsP_3$ generated. $InsP_3$ interacts with receptors ($InsP_3R$) in the sarcoplasmic-reticulum membrane to stimulate Ca^{2+} release. $InsP_3$ also enters the nucleus, where it interacts with $InsP_3R$, leading to Ca^{2+}-mediated activation of intranuclear CAMKII. This, in turn, activates PKD, resulting in the phosphorylation of HDAC and its subsequent nuclear export and thereby altering transcriptional regulation. Pools of intracellular Ca^{2+} also activate cytosolic calmodulin–CAMKII, resulting in the activation of NFAT. Activated NFAT then translocates to the nucleus, where it is involved in transcriptional regulation. Therefore, in the failing heart, normal calcium cycling becomes dysregulated by multiple abnormalities.

The density of capillaries in the heart muscle is another important parameter that affects energy availability. In mice with hypertrophy as a result of cardiac-specific overproduction of Akt, pathological cardiac remodelling is associated with an inability of angiogenesis to keep up with muscle growth[84]. The transcription factor GATA4 has an important stimulatory role in angiogenesis and thus helps to maintain the balance between muscle growth (hypertrophy) and nutrient supply (capillary density)[85]. The hypertrophic stress of pressure overload initially stimulates expression of the gene encoding hypoxia-inducible factor 1α (HIF1α), a key stimulator of angiogenesis; however, subsequent upregulation of production of the tumour-suppressor protein p53 suppresses HIF1α activity in later stages of remodelling, leading to a dilated cardiac phenotype[86]. Thus, targeted inhibition of antiangiogenic factors such as p53 might benefit patients with ischaemic cardiomyopathy.

The sarcomere

The beating of the heart depends ultimately on the force generated by the sarcomere, through the interaction of thick filaments, which are composed of myosin, with actin-containing thin filaments. For inherited cardiomyopathies (whether they have a hypertrophic or a dilated phenotype), mutations in genes encoding sarcomere proteins constitute most of the known genetic causes[20]. On the basis of studies using mice that produce these mutant forms of sarcomere proteins, insights into structure–function relationships have helped to identify key amino-acid residues that regulate contractile strength and relaxation.

In heart-failure models in mice and rats, the expression ratio of the α-myosin heavy chain and β-myosin heavy chain shifts, and this can have a major role in altering the contractile phenotype. Adult rodent hearts normally contain α-myosin heavy chain, which has faster crossbridge kinetics but generates less tension than the fetal protein, β-myosin heavy chain. In the setting of cardiac hypertrophy or failure, however, the gene encoding β-myosin heavy chain is re-expressed, contributing up to half the total amount of myosin heavy chain. The hearts of humans (and of all large mammals) contain β-myosin heavy chain nearly exclusively, but changes can still occur in failing hearts, with further reductions in expression of α-myosin heavy chain[87]. Efforts to increase expression of α-myosin heavy chain, potentially through modulation of miR-208 (which is encoded by intron 27 of the gene encoding α-myosin heavy chain and seems to regulate the switch in gene expression[49]), might yield a useful therapeutic intervention.

The sarcomere contains many proteins other than actin and myosin (Fig. 4). These include regulatory thin filaments (which consist of troponins and α-tropomyosin), interlinking proteins (myosin-binding protein C and titin) and a protein complex (in the Z-disk) that couples mechanical forces to signalling by protein kinases and phosphatases. For example, calsarcin 1 — present in the Z-disk — is an anchoring protein for calcineurin. Calsarcin 1 interacts with titin and responds to stretch activation, functioning to negatively regulate calcineurin signalling at the Z-disk[71]. The proteins titin cap (T-cap; also known as telethonin) and muscle LIM protein (MLP; also known as CLP) also interact with titin at the Z-disk and are thought to form a mechanical stretch receptor that affects sarcomere contraction. Mutations in the gene encoding T-cap that promote the association of T-cap with titin and calsarcin 1 result in hypertrophic cardiomyopathy, whereas mutations that impair this association result in dilated cardiomyopathy, as do mutations in the gene encoding MLP[88,89]. Manipulating these interactions might ultimately provide a new approach to the treatment of heart failure.

Titin has garnered attention as an important regulator of myocardial mechanosignalling and structural stiffness. It functions as a molecular spring that extends from the M-line to the Z-line and is required for sarcomere assembly[90]. The distensibility of titin is provided by a PEVK region (which is rich in proline, glutamic acid, valine and lysine residues). There are a short isoform and a long isoform of the protein (known as N2B and N2BA, respectively), and these have PEVK regions of different lengths, resulting in different distensibilities. In failing hearts, the relative amount of the two isoforms changes (with the exact ratio varying depending on the disease condition), and this change correlates

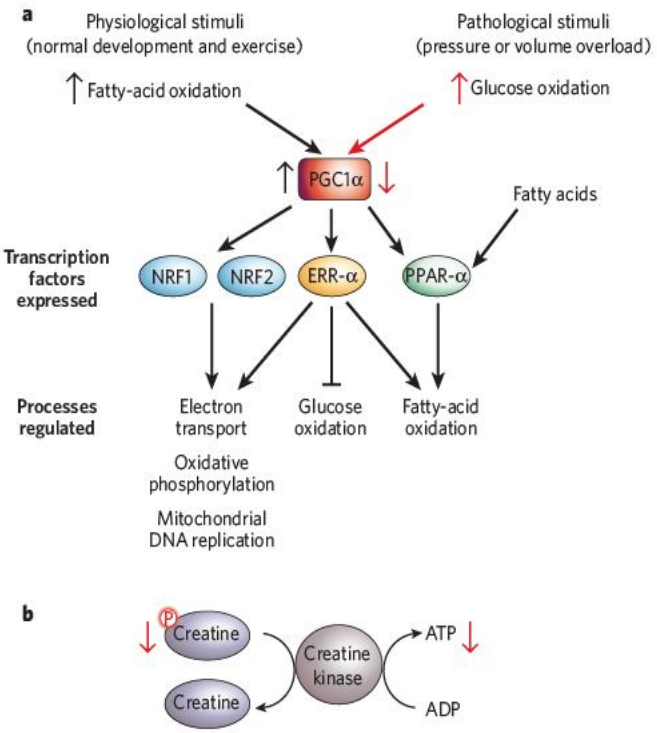

Figure 3 | Abnormal metabolism and energy regulation in the failing heart. **a**, In the normal heart, there is a preference for oxidation of fatty acids over glucose. In the failing heart, there can be a shift towards increased glucose oxidation, which is coupled to a decline in production of the transcriptional coactivator PGC1α. PGC1α modulates the expression of the transcription factors PPAR-α, ERR-α, NRF1 and NRF2, which affect mitochondrial biogenesis and fatty-acid oxidation. **b**, Creatine kinase generates ATP from phosphocreatine and ADP. In the failing heart, creatine kinase has reduced activity, and there is a decline in the ratio of phosphocreatine to ATP.

with diastolic distensibility[91]. Titin also contains a kinase domain that can couple mechanical stimuli to downstream signalling. In mice, deletion of the portion of the gene encoding this domain results in decreased contractility and abnormal handling of Ca^{2+}, leading to hypertrophy and heart failure[92]. Sarcomere proteins such as titin are crucial targets of various protein kinases and phosphatases, and manipulating their post-translational modification could provide a new tool for increasing chamber contraction and improving diastolic function.

The treatment of heart failure using agents to increase contractile force has been problematic (as discussed earlier), as this often leads to reduced function. However, new insights into the regulation of myofilament Ca^{2+} sensitivity have led to efforts to increase contractility without affecting the cellular Ca^{2+} concentration or the energy demand imposed on the heart, so prospects for such treatments might improve. Agents that stimulate myosin ATPase activity, interact with cysteine residues to alter protein function, or modulate the interaction of Ca^{2+} with troponin C can increase contractility and are in development[93].

The bionic heart

Despite the many recent advances in understanding the molecular and cellular mechanisms that underlie heart failure, the most notable new treatment in the past decade is the use of implantable pacemakers to achieve cardiac resynchronization. This therapy is used in patients with an intraventricular conduction delay, which results in uncoordinated wall motion and inefficient contraction. By simultaneous electrical stimulation of the left and right ventricles, the synchrony of left ventricle contraction can be restored, and the mechanoenergetics of the heart can be improved, both of which lead to improvements in clinical symptoms and reduced patient mortality[94]. In animals, cardiac resynchronization therapy reverses regional activation of stress-activated kinases in the walls of dyssynchronously contracting hearts and is associated with a global improvement in cell survival that is mediated by Akt–BAD

(Bcl-2-antagonist of cell death) signalling[95]. At present, however, about 30% of patients who receive cardiac resynchronization therapy do not benefit clinically. It might be possible to reduce the non-responder rate by more accurately assessing both the electrical activation delay and the mechanical discoordination that is present. Abnormalities in one do not automatically imply abnormalities in the other, and more insight is needed into the role of each in determining who responds to this therapy.

Another important type of implantable device is the implantable cardioverter defibrillator (ICD). The ICD was initially developed to treat patients with ischaemic heart disease who had suffered from ventricular arrhythmias and were at risk of sudden cardiac death. However, the role of ICDs was greatly expanded after reports that they reduce the incidence of mortality when implanted prophylactically in at-risk patients who have not yet had episodes of arrhythmia[96]. Although individuals at risk can be identified, it is difficult to predict who will actually require ICD therapy. Given the cost and complexity of the device, it is important to improve patient selection, and research efforts are underway to achieve this by using electrophysiological, molecular and genomic analyses.

Although the totally implantable artificial heart is far from realized, an alternative that supports only left ventricular function (the left ventricular assist device, LVAD) has become a mainstay of therapy. Studies of myocardium from patients before and after they have received an LVAD show that this therapy results in remarkable improvements in Ca^{2+} cycling, adrenergic responsiveness, and molecular and morphological remodelling. These studies have provided much of the current understanding of the molecular and cellular plasticity of human heart failure[97]. The findings have led some researchers to propose that LVAD therapy might result in true myocardial recovery, and data from a clinical trial combining LVAD implantation with concurrent β_2-AR-agonist treatment (to promote a physiological form of hypertrophy) suggest that such recovery might be attainable[98].

Other approaches have been developed, in which electrical stimulation is used to alter heart function without triggering a contraction. A therapy termed non-excitatory cardiac contractility modulation involves application of a high-frequency (10-Hz) electrical pulse to the heart during a specific window of the heartbeat (the electrical refractory period). The heart then responds with improved function. Although some evidence points to changes in Ca^{2+} handling, the precise mechanisms underlying this improved function remain unclear[99]. Another approach is stimulation of the vagus nerve in the neck, a procedure that can improve post-infarction remodelling and can suppress heart-failure development, despite there being no change in heart rate[100]. Such neural modulation might ultimately prove to be a potent therapy for heart failure.

The future

Knowledge of the pathophysiology of heart failure is growing rapidly, and many new therapeutic targets have been identified. But the

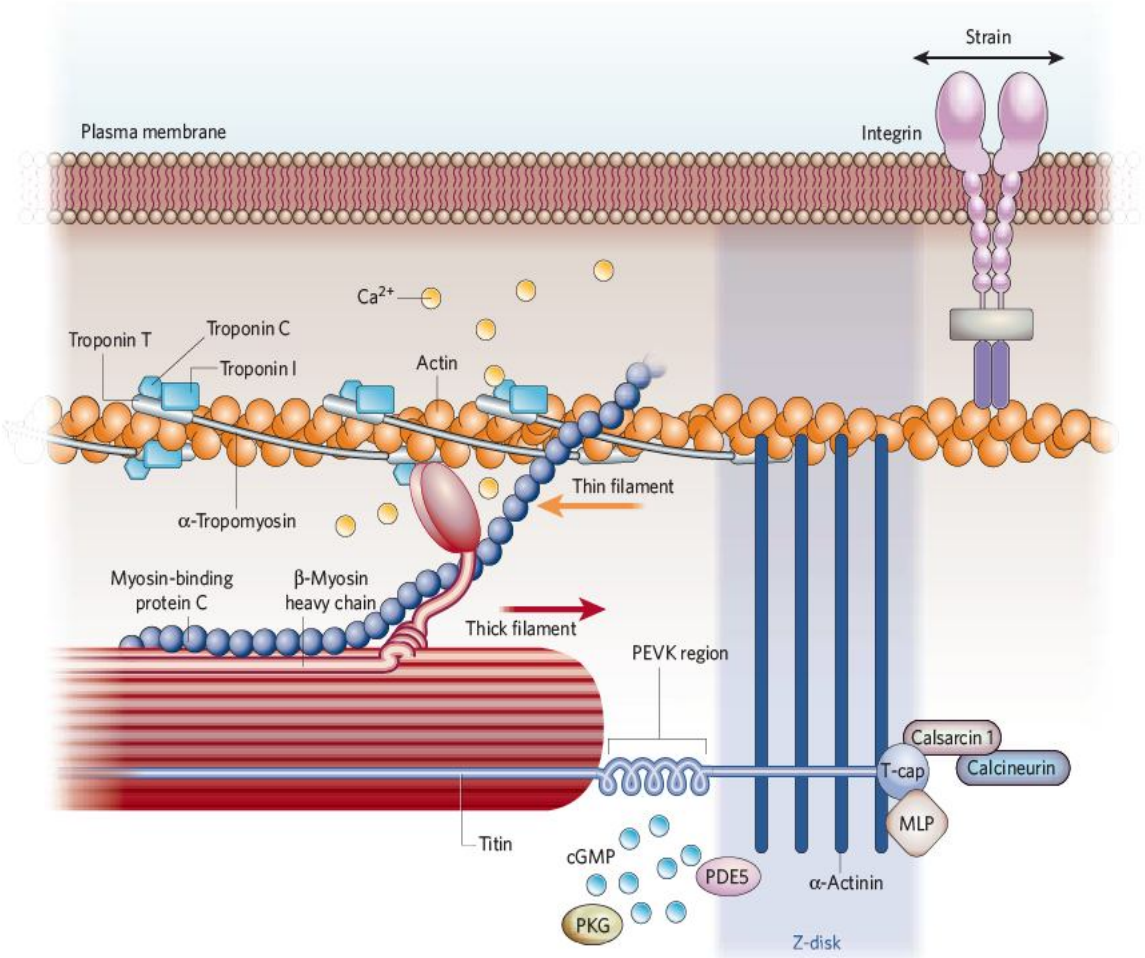

Figure 4 | Modulation of contraction by regulatory myofilament proteins in a cardiomyocyte. Mechanical stimuli are transduced by clustered membrane integrins (in association with accessory proteins, such as the dystrophin-associated protein complex; not shown in detail) that couple to the Z-disk of the sarcomere (shaded). Proteins such as calsarcin 1, MLP and titin cap (T-cap) are localized to the Z-disk. These proteins couple the input from the integrin to the contractile (thick and thin) filaments by interacting with α-actinin, titin, actin and other proteins. Ca^{2+} interacts with troponin C, resulting in a conformational change in troponin I. This, in turn, releases α-tropomyosin from its position, in which it prevents actin from binding to myosin. The result is the formation of force-generating crossbridges. Thin-filament regulatory proteins (namely troponin T, troponin C, troponin I, myosin-binding protein C and α-tropomyosin) and titin can be post-translationally regulated by protein kinases and/or phosphatases (not shown). For example, PDE5 is present in the Z-disk and might regulate local pools of cGMP, which could then activate PKG. This, in turn, could reduce the sensitivity of myofilaments to Ca^{2+} and thereby depress contraction. In the failing heart, the post-translational modification of titin seems to be an important mechanism leading to contractile dysfunction.

potential of few of these molecular targets has been realized. It is hoped that this situation will change soon, as mechanistic insights are translated into therapies that improve the clinical symptoms and lengthen the lifespan of patients with heart failure. The threshold of implanting a microprocessor that senses abnormal heart function and delivers therapy (at present, electrons; and in the future, perhaps drugs) has already been crossed. Advances in microengineering and sensor technologies are likely to expand the potential of these devices. Cell-based and gene therapies also hold promise — cell-based therapy can potentially stave off myocardial loss or even regenerate the myocardium, and gene therapy can offset molecular abnormalities that cannot be targeted with small molecules. So, although the description of heart-failure symptoms has changed little since the twelfth century, the strategies for combating it are developing rapidly, and there is now the potential to markedly reduce the prevalence of this disease in the twenty-first century. ■

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A genetic framework for improving arrhythmia therapy

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Abnormalities in heart rhythm continue to cause high rates of illness and death. Better treatment could be provided by solving two main challenges: the early identification of patients who are at risk, and the characterization of molecular pathways that culminate in arrhythmias. By analysing mechanisms that increase susceptibility to arrhythmia in individuals with genetic syndromes, it might be possible to improve current therapies and to develop new ways to treat and prevent common arrhythmias.

Abnormal heart rhythms, notably atrial and ventricular fibrillation, are leading causes of death and disability. Between 0.5 million and 1 million North Americans and Europeans die each year from sudden cardiac death (SCD), which causes 10–20% of all deaths among adults in the Western world[1,2]. The most common underlying cause is ventricular fibrillation (Fig. 1). SCD most often occurs in patients with underlying coronary artery disease. Hence, as the prevalence of the disease increases worldwide, so will the incidence of SCD. Evaluation of patients who are known to have heart disease can identify those at increased risk of SCD, but unfortunately in about half of all cases SCD is the initial presenting symptom of heart disease[2,3]. Among patients judged to be at very high risk of SCD, implantable cardioverter defibrillators (ICDs) are often used (Box 1). These devices are highly effective at terminating an otherwise fatal arrhythmia if it occurs, but they do not prevent arrhythmia.

Atrial fibrillation affects more than 2 million North Americans and causes substantial morbidity and mortality, predominantly through stroke, heart failure, and disabling symptoms such as palpitations and dizziness. Current therapies for atrial fibrillation comprise either symptomatic control by decreasing the heart rate during episodes of atrial fibrillation or intervention — using drugs or ablation therapy — that can prevent arrhythmia from occurring[4] (Boxes 2 and 3). However, the available drugs are incompletely effective, and such treatment carries risks, including actually promoting arrhythmia (Box 2).

There are many other forms of abnormal heart rhythm, but atrial and ventricular fibrillation stand out because they are common and serious forms of arrhythmia and because safe and effective preventive therapies have not yet been developed. Here we focus on how the emerging understanding of underlying mechanisms, especially in rare genetic arrhythmia syndromes, might inform the development of such therapies. In particular, we emphasize how identifying the molecular and biophysical abnormalities that cause arrhythmias can help to define pathways and molecules that can be targeted to prevent arrhythmia.

Arrhythmia and electrophysiological abnormalities

The normal heartbeat is driven by highly choreographed membrane depolarization and repolarization in single heart cells (Fig. 2), propagating in an orderly manner from sinus node to atrium to ventricle. Mutations that alter the normal function of even a single component of this system can create a highly arrhythmogenic substrate. The development of antiarrhythmic drugs has been difficult because the underlying mechanisms of arrhythmia have not been well defined and because most drugs have targeted components of normal electrophysiology (Box 2). Using drugs to 'tinker' with a complex biological system runs the risk of creating an abnormal electrophysiological substrate, thereby failing to be antiarrhythmic or, worse still, actually promoting arrhythmia[5]. Given this complexity, an alternative approach is to design drugs that target the specific abnormal process that leads to arrhythmia in the individual (Table 1).

Arrhythmias occur because of abnormalities either in the electrophysiology of individual cells or in cell-to-cell propagation. One common arrhythmia mechanism is abnormal automaticity: that is, rapid pacemaker-like rhythmic beating in cells that do not usually demonstrate such behaviour. The mechanisms for such abnormal focal arrhythmic activity are not completely understood, but the evidence supports a prominent role for abnormal cycling of intracellular calcium ions (Ca^{2+}). In some instances, focal activity starts spontaneously, whereas in others a preceding normal action potential is required as a 'trigger'. Triggered rhythms are commonly subdivided into those occurring when the antecedent heart rate is slow (for example, long QT-interval-associated arrhythmias) and when it is rapid (for example, catecholaminergic polymorphic ventricular tachycardia).

Another common arrhythmia mechanism is re-entrant excitation: that is, continuous excitation in an electrical circuit without independent triggering of each beat. Re-entrant circuits can involve a small region of heart tissue (a condition known as micro-re-entry), or they can involve large regions in a single chamber or in several chambers (macro-re-entry). Heterogeneity in the electrophysiological properties of tissue in the circuit, notably differences in refractory periods or in conduction velocity, promotes re-entry. Sometimes the re-entrant circuit is anatomically fixed, and in these cases ablation at a key location within the circuit can cure the patient (Box 3). Fibrillation — atrial or ventricular — is a subtype of re-entry in which there is no fixed circuit; rather, each cell is excited by neighbouring cells as soon as it has repolarized, so orderly impulse propagation and contraction are absent. The two arrhythmia mechanisms — abnormal automaticity and re-entrant excitation — are not mutually exclusive. For example, abnormal automatic behaviour in atrial cells can initiate atrial fibrillation in the susceptible heart[6]. Some heterogeneities of cardiac electrophysiology occur normally; however, exaggeration of such heterogeneities by drug administration, genetic lesions or changes in heart rates can promote arrhythmias.

The arrhythmia-prone heart

Arrhythmias reflect an interaction between a susceptible substrate (for example, an anatomically defined circuit, a myocardial scar, atrial

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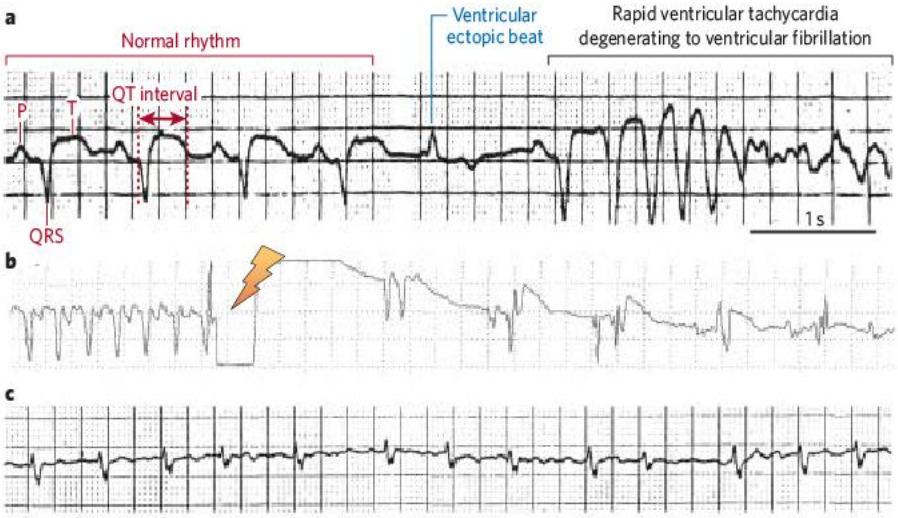

Figure 1 | Abnormal heart rhythms. **a**, Cardiac rhythm recorded in a patient who died as a result of SCD. The four beats on the left demonstrate the patient's normal rhythm. Components of the normal electrocardiographic complex are indicated in red: each beat starts with a P wave, representing normal atrial activity, and is followed by a QRS complex, representing ventricular depolarization. Ventricular repolarization is shown on the electrocardiogram by the QT interval: that is, the time from the onset of the QRS wave to the end of the repolarization (T) wave that follows (indicated by the horizontal arrow). In this case, the morphology of the T wave suggests active myocardial ischaemia. A ventricular ectopic beat (indicated in blue) is followed by fast ventricular tachycardia that degenerates to ventricular fibrillation. The calibration bar (1 s) applies to all panels. **b**, Intracardiac recording retrieved from an ICD in a patient with ischaemic heart disease. On the left, the tracing shows fast ventricular tachycardia, and this is followed by delivery of a shock (jagged arrow) and subsequent restoration of the patient's normal rhythm. **c**, Atrial fibrillation. Rather than discrete P waves at the beginning of each complex, the baseline is undulatory, and the overall rate is irregular.

fibrosis or a monogenic arrhythmia syndrome) and a triggering event (such as adrenergic surge, inflammation, an episode of acute myocardial ischaemia, a change in wall tension due to stretch, or administration of a drug).

A very small number of patients are susceptible to arrhythmias because they are born with accessory atrioventricular pathways, known as bypass tracts. In these cases, the most common type of arrhythmia is a regular rapid heart beat (tachycardia) generated by conduction from atrium to ventricle over the normal atrioventricular nodal pathway and re-entry to the atrium using the bypass tract. Patients with accessory pathways frequently have abnormal electrocardiograms even during normal rhythm (known as Wolff–Parkinson–White syndrome) and can be cured by ablation of targeted portions of the re-entrant circuit (Box 3).

In some cases — particularly in many monogenic arrhythmia syndromes (Table 1) — a susceptible substrate can be present even in the absence of any structural heart disease detectable by conventional techniques, such as echocardiography or magnetic resonance imaging. In the case of atrial fibrillation, about one-third of patients have 'lone' atrial fibrillation: that is, they develop the arrhythmia when young (before 60 years of age), and they lack known risk factors and structural heart disease[7,8]. More commonly, existing heart disease results in changes that constitute the arrhythmia-prone substrate, including changes in electrophysiological or contractile function, changes in cardiomyocyte–cardiomyocyte connections, or extracellular fibrosis. Mottled scarring following acute myocardial infarction (heart attack) can generate the substrate for ventricular tachycardia or ventricular fibrillation[9], and hypertension and age-related fibrosis are common in those with atrial fibrillation[10–13].

The presence of heart disease, or indeed frequent episodes of arrhythmia itself, can result in a remodelling process that further increases susceptibility to arrhythmia. A seminal observation in the mid-1990s was that rapid atrial pacing increases susceptibility to atrial fibrillation after pacing has stopped. It was found that atrial fibrillation was more readily elicited and lasted longer when the duration of antecedent pacing was increased; so "atrial fibrillation begets atrial fibrillation"[14]. This finding provoked research into the underlying mechanisms and raises the possibility that early definitive intervention to prevent remodelling associated with atrial fibrillation could make therapies more effective. Extensive study of experimental atrial fibrillation suggests that there is an early phase of remodelling during rapid atrial excitation, probably reflecting a decrease in the number of cell-surface L-type Ca^{2+} channels[15,16]. This remodelling is followed by altered expression of these and other channels, as well as of other proteins (for example, those in

Box 1 | Implantable cardioverter defibrillators

Implantable cardioverter defibrillators (ICDs) are devices that use pacemaker technology to identify abnormally rapid rhythms and to terminate these promptly (Fig. 1b). This approach was developed almost 30 years ago. Initially, the devices were bulky and required thoracotomy (which had a 1–2% mortality rate) for implantation, so they were reserved for patients judged to be at very high risk of SCD. Technological advances have now resulted in small devices that are readily implanted and have sophisticated diagnostic, therapeutic and recording capabilities. Current devices do have complications, including the potential for painful shocks in response to non-life-threatening arrhythmias, infection, and generator and lead malfunction, but these occur rarely.

ICDs were initially only used in patients who had been resuscitated after an episode of SCD caused by ventricular fibrillation (that is, as secondary prevention)[76]. More recently, randomized, controlled clinical trials have supported the use of ICDs in patients who are at increased risk of SCD — notably those with heart disease and decreased contractile function — even in the absence of a past history of arrhythmia (primary prevention)[77,78]. This is a much larger group of patients, and more ICDs must be implanted to prevent a single case of SCD[79]. Although ICDs clearly prevent SCD in some patient populations, widespread deployment is expensive. Moreover, SCD is the presenting symptom of heart disease in more than half of all affected subjects[2,79]. Therefore, studies are needed to improve the identification of patients who are not yet known to have heart disease but are at high risk of SCD.

Patients with severe contractile dysfunction are also at a high risk of SCD. In this group, the insertion of devices that have ICD capabilities, and the ability to pace at multiple ventricular sites to improve contractile function, can improve symptoms and longevity in some patients[80].

Box 2 | History of therapy with current antiarrhythmic drugs and their limitations

Drugs that are currently marketed as antiarrhythmics were developed in whole-animal models by screening for effects on normal cardiac electrophysiology. Indeed, decades elapsed between the initial use of older antiarrhythmic drugs in humans and the definition of their molecular mechanisms of action. Antiarrhythmic drugs are only partly effective and have many adverse effects, most importantly the potential to generate new life-threatening arrhythmias (a phenomenon known as proarrhythmia).

The ability to record cardiac rhythm over long periods led to the observation in the 1970s that patients with frequent ventricular ectopic beats (Fig. 1) after an acute myocardial infarction were at increased risk of SCD. But testing the hypothesis that suppressing such isolated abnormal beats would improve survival was not possible, because older antiarrhythmics produced a very high incidence of non-cardiac side effects, such as gastrointestinal intolerance or allergic reactions. The development in the 1980s of well-tolerated drugs that suppressed ectopic beats both in experimental animals and in humans led to the National Heart Lung and Blood Institute's Cardiac Arrhythmia Suppression Trial (CAST). CAST was terminated prematurely in 1989 when therapy with these 'well-tolerated' drugs was found to increase mortality twofold to threefold compared with that in patients treated with a placebo[81].

This study was important not only for antiarrhythmic drug development but also for drug development in general. It reinforced the importance of a randomized, placebo-controlled trial with a primary 'hard' end point such as death — as opposed to a surrogate end point, such as ectopic-beat suppression — to sort out whether a therapy is beneficial or not. The drugs tested in CAST turned out to be potent sodium (Na^+)-channel blocking agents. It is thought that blocking Na^+ channels increases SCD risk by slowing conduction or by increasing the heterogeneity of repolarization, both of which can be arrhythmogenic, especially in a diseased heart[82–84].

Another potential mechanism for arrhythmia suppression, prolonging cardiac refractoriness without targeting Na^+ channels, was intensely investigated and developed after CAST. The most common molecular mechanism to achieve this effect is blocking the rapid component of the cardiac delayed rectifier potassium (K^+) current, I_{Kr}, encoded by *KCNH2* (also known as *HERG*). However, blocking I_{Kr} carries the risk of marked QT-interval prolongation and a distinctive form of polymorphic ventricular tachycardia, termed torsades de pointes. Indeed, K^+-channel-blocking antiarrhythmic drugs have been tested in CAST-like trials and did not prevent more deaths than a placebo[85–87]. Moreover, the same mechanism underlies the development of torsades de pointes in response to 'non-cardiovascular' drugs such as certain antihistamines, antibiotics and antipsychotics[88]. I_{Kr} blockers lead to increased action potential duration, allowing the activation of arrhythmogenic inward currents (transduced by entirely normal molecular mechanisms, such as those mediated by L-type calcium (Ca^{2+}) channels or Na^+/Ca^{2+} exchangers), thereby generating arrhythmias.

Thus, drugs acting on a single molecular target — either Na^+ channels or KCNH2 channels — in a complex biological environment such as the cardiomyocyte (Fig. 2) might have unintended electrophysiological consequences. As a corollary, the development of antiarrhythmic drugs that target activation of abnormal arrhythmogenic pathways — which are absent or minimally active in normal tissue — could be a safer and more effective route to drug development (Table 1). Indeed, β-blockers, which do not directly target ion channels, have not shown serious proarrhythmic effects and can reduce the incidence of SCD[89,90]. In addition, animal experiments and clinical trials have identified other pharmacological interventions that seem to have antiarrhythmic properties but do not target ion channels[91]: inhibitors of the renin–angiotensin system (angiotensin-converting-enzyme inhibitors and angiotensin-receptor blockers)[92–94], HMG-CoA-reductase inhibitors (statins)[95,96] and fish oil[97–99]. The mechanisms by which these interventions, which are not reported to have proarrhythmic potential, exert antiarrhythmic effects is an area of intense investigation, because identification of the responsible molecular pathways could lead to the development of more specific, effective and safe antiarrhythmic drugs.

the extracellular matrix), which together generate the atrial-fibrillation-prone substrate[17–22]. Rapid ventricular rates in atrial fibrillation can also remodel the ventricle to increase susceptibility to arrhythmias and decrease contractile function[23]; indeed, rapid ventricular pacing is a commonly used approach to generate heart failure in animals[24,25]. In addition, other forms of remodelling, such as ventricular hypertrophy in normal tissue adjacent to a myocardial infarction, can generate an arrhythmia-prone substrate[26].

Genetics of arrhythmia susceptibility

In the 1950s, a familial disease characterized by an unusual electrocardiogram (with QT-interval prolongation), a structurally normal heart and an increased risk of SCD was first described. The long-QT syndromes (LQTS) and other rare genetic diseases that cause arrhythmias remained electrophysiological curiosities until the accumulation of large numbers of kindreds from registries allowed a comprehensive description of clinical features and, most importantly, the identification of disease-associated genes. In parallel research, familial diseases in which patients presenting with cardiomyopathy were described, and disease-associated genes were identified. All familial cardiomyopathy syndromes are associated with increased susceptibility to arrhythmias, and in many cases arrhythmias such as atrial fibrillation, SCD due to ventricular fibrillation, or profound bradyarrhythmia (an abnormally slow heart rate) can be the presenting symptoms that lead to the diagnosis of heart disease in the patient or family. (See refs 27–29 for a detailed discussion of specific types of inherited arrhythmia syndrome.)

These diseases have several common features that have important implications for the diagnosis and therapy of arrhythmia. First, these syndromes are more common than was once appreciated: early estimates of LQTS prevalence of 1 in 10,000 have been replaced by estimates of 1 in 1,000–2,000 (ref. 30). Second, the identification of disease-associated genes provides an important starting point for studies of molecular mechanisms of common arrhythmias. Third, elucidation of these mechanisms might lead to the identification of genetic markers of increased risk of atrial fibrillation or SCD in the population. Knowledge of genetic variants might also be useful for identifying patients who are likely to respond particularly well or poorly to drugs or other therapies. Fourth, insight into these mechanisms also provides a strong argument for avoiding commercialization of certain drug actions. For example, loss-of-function mutations in the genes whose expression underlies I_{Kr} and I_{Ks} (key potassium ion (K^+) currents in the heart) cause LQTS; drugs that block these currents phenocopy the congenital

Box 3 | Some arrhythmias can be cured

Intensive work over the past 30 years has developed technologies for identifying stable re-entrant circuits responsible for arrhythmias. A very high proportion (more than 95%) of patients with such circuits but with structurally normal hearts can be cured by delivery of ablative energy to highly selected regions of these circuits, resulting in elimination of the arrhythmia[100]. In other patients, re-entrant circuits arise as a consequence of underlying heart disease (for example, certain forms of ventricular tachycardia after myocardial infarction); in these cases, ablation can eliminate one cause of arrhythmia but often leaves the arrhythmogenic substrate intact, so these patients continue to be at risk of recurring arrhythmias.

More recently, catheter-based mapping has been used to identify foci of abnormal electrical activity that drive atrial fibrillation from within pulmonary veins[6]. Ablation applied directly to the foci can eliminate the arrhythmia but carries a substantial risk of pulmonary vein damage. Thus, current techniques use 'catheter ablation' not to ablate the foci themselves but to isolate such foci from the remainder of the atrium, thereby preventing propagation from the foci. Patients undergoing this type of ablative therapy for atrial fibrillation have a lower cure rate than those with a stable arrhythmia circuit that can be targeted by ablation.

disease and thus can be proarrhythmic (Table 1). Last, these common features strongly reinforce the idea that arrhythmia therapies are most effective when targeted to underlying pathophysiological mechanisms. This concept was enunciated almost 20 years ago by a Task Force of the Working Group on Arrhythmias of the European Society of Cardiology and is known popularly as the Sicilian Gambit[31]. Table 1 extends this concept to suggest mechanism-based molecular therapies for congenital and acquired diseases associated with increased risk of arrhythmia. Thus, whereas blocking I_{Kr} or I_{Ks} has the potential to be deleterious in most patients, there is a small subset of people with arrhythmias caused by gain-of-function mutations in the underlying genes. In those individuals, K^+-channel blockers might be curative. The identification of genetically defined subtypes of risk of atrial fibrillation or SCD opens the door to personalizing therapy by targeting the specific causative mechanism in an individual patient.

Common features of monogenic arrhythmia syndromes

With few exceptions, monogenic arrhythmia syndromes are transmitted in an autosomal dominant manner with variable penetrance. Occasional individuals who inherit two abnormal disease-associated alleles (often from parents who have minimal clinical phenotypes) have a much more severe phenotype than individuals with one disease-associated allele. In general, monogenic arrhythmia syndromes in patients with structurally normal hearts can be viewed as 'diseases of the action potential', with mutations affecting mainly ion channels, although there are exceptions. Similarly, hypertrophic cardiomyopathy can be viewed as a disease of the sarcomere, dilated cardiomyopathy a disease of the cardiac cytoskeleton, and arrhythmogenic right ventricular cardiomyopathy a disease of cardiomyocyte–cardiomyocyte adhesion.

Almost all studies in the field have focused on mutations in the germ line. However, it is also possible that somatic mutations might predispose individuals to arrhythmias. In one small case series of patients with atrial fibrillation, a mutation was detected in *GJA5* — which encodes the gap junction protein connexin 40 — in atrial cells but not in the germ line[32]. The role of such somatic mutations in determining arrhythmia susceptibility in humans remains otherwise unexplored.

Despite these unifying themes, the details of the clinical phenotype — which might be important for clinical management and prognosis — vary not simply by broad disease category but by disease-associated gene. Thus, in patients with certain forms of LQTS, SCD occurs predominantly during exercise. By contrast, for other forms of LQTS, it occurs predominantly when patients are at rest[27]. Similarly, some studies suggest that mutations in myosin-binding protein C tend to cause symptoms later in life in patients with hypertrophic cardiomyopathy than do mutations affecting other genes[33]. In addition, when large numbers of families have been studied, mutations affecting specific protein domains or specific amino-acid residues have been implicated in a better or worse prognosis[34–36]. The idea that disease prognosis and management could vary with

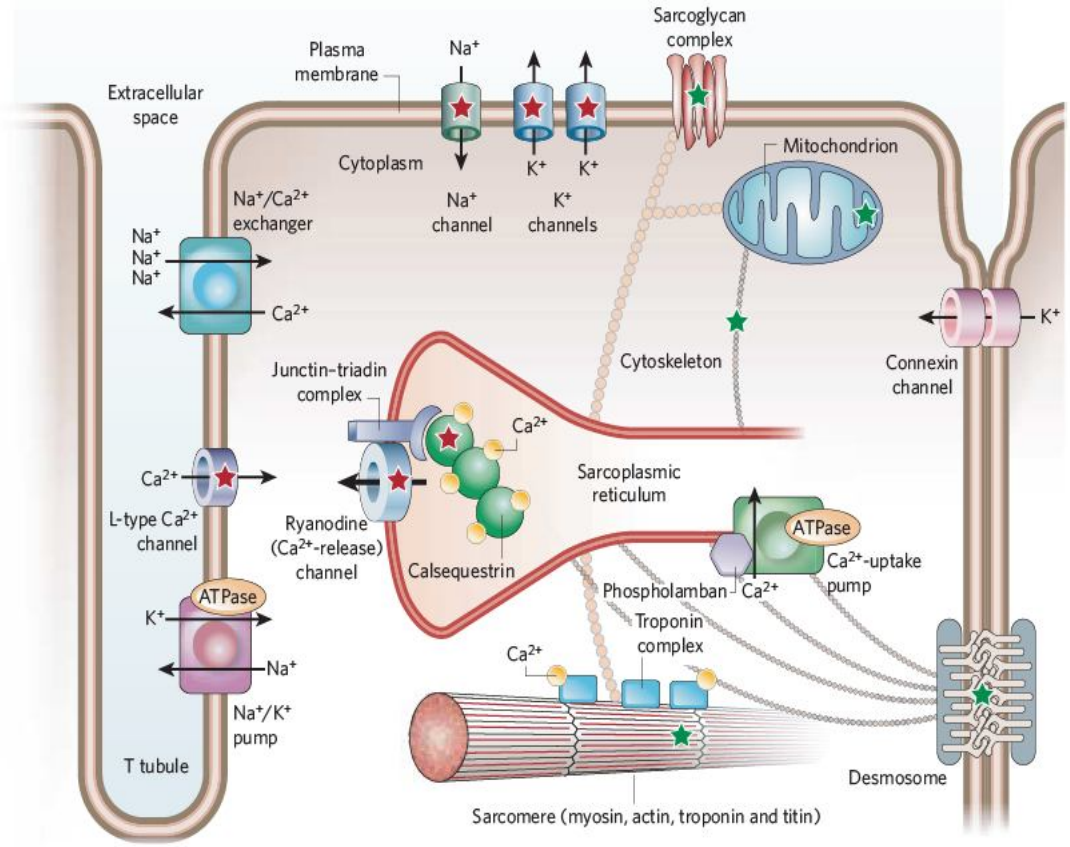

Figure 2 | A ventricular cardiomyocyte. Illustrated are the protein complexes, cardiomyocyte architecture and intracellular organelles involved in cardiac excitation–contraction coupling. The initial event in the cardiac cycle is membrane depolarization, which occurs with ion entry through connexin channels from a neighbouring cardiomyocyte (right) followed by opening of voltage-gated Na^+ channels and Na^+ entry (top). The resultant rapid depolarization of the membrane inactivates Na^+ channels and opens both K^+ channels and Ca^{2+} channels. Entry of Ca^{2+} into the cell triggers the release of Ca^{2+} from the sarcoplasmic reticulum through the ryanodine channel. Ca^{2+} then binds to the troponin complex and activates the contractile apparatus (the sarcomere, bottom). Cellular relaxation occurs on removal of Ca^{2+} from the cytosol by the Ca^{2+}-uptake pumps of the sarcoplasmic reticulum and by Na^+/Ca^{2+} exchange with the extracellular fluid. Intracellular Na^+ homeostasis is achieved by the Na^+/K^+ pump. The molecular components that are required for normal electrophysiological activity, contractile function and cell–cell adhesion (the latter mediated by desmosomes) all need to be positioned correctly within the cell and anchored to each other and the cytoskeleton. Some cardiomyocyte components are not shown (for example, stretch-activated channels, and ankyrins that target channels and other proteins to their correct locations within the cell). Red stars indicate proteins encoded by genes that are mutated in primary arrhythmia syndromes; many of these proteins form part of macromolecular complexes, so mutations in several genes could be responsible for these syndromes. Green stars indicate protein complexes in which mutations in multiple genes cause cardiomyopathies often associated with arrhythmias; these complexes include the sarcomere (in hypertrophic cardiomyopathy), the desmosome (in arrhythmic right ventricular cardiomyopathy), and the cytoskeleton, sarcoglycan complex and mitochondrion (in dilated cardiomyopathy).

Table 1 | A mechanism-based approach to antiarrhythmic drug therapy

Mechanism	Phenotype			Disease-associated genes	Possible acquired syndrome	Potential mechanism-specific drug therapy
	Molecular	Cellular	Clinical			
Failure of physiological channel closing or inactivation	Destabilized Na^+-channel inactivation	Action potential prolongation, EADs	Long QT interval (most prominent at rest)	*SCN5A*, *CAV3*, *SCN4B*	Drug-induced LQTS	Blocker of Na^+ channels
	Destabilized ryanodine (Ca^{2+}-release) channels	DAD-mediated automaticity, premature Ca^{2+} release from SR	Catecholaminergic polymorphic VT	*RYR2*	Arrhythmia in heart failure, phenocopied by digitalis intoxication	Stabilizers of ryanodine channels; blocker of Ca^{2+}-activated arrhythmogenic processes
			Atrial and ventricular arrhythmia, bradycardia	*CASQ2*		
	Abnormal L-type Ca^{2+}-channel inactivation	Action potential prolongation, EADs	Timothy syndrome (LQTS and extracardiac phenotypes include syndactyly and abnormal facies)	*CACNA1C*	Drug-induced LQTS	Blocker of Ca^{2+} channels
Increased repolarizing K^+ current	Failure of fast I_{Kr} inactivation	Action potential shortening	SQTS (characterized by AF and VF)	*KCNH2*	AF induced by vagal stimulation	Blocker of affected K^+ channel
	Negative shift in voltage dependence of I_{Ks} activation			*KCNQ1*		
	Increased outward (repolarizing) current due to failure of normal inward rectification			*KCNJ2*		
Increased non-selective stretch current	ND	Action potential shortening, DADs	AF	ND	AF	Blocker of stretch-activated channels (such as spider toxin MTx-4)
Decreased current as a result of abnormal channel gating or decreased cell-surface protein expression	Decreased Na^+ current	Loss of action potential dome in epicardial cells	Increased PR and QRS duration (slow conduction), ST-segment elevation, VF (Brugada syndrome)	*SCN5A*	SCD during therapy with Na^+-channel blockers, especially in ischaemic heart disease	Activator of peak Na^+ current; blocker of transient outward current
		Decreased upstroke slope of action potentials	Isolated conduction-system disease (PR and QRS prolongation, bundle branch block, AV nodal block)			
	Decreased K^+ current	Action potential prolongation, EADs	LQTS (often with exertion)	*KCNQ1*, *KCNH2*, *KCNE1*, *KCNE2*, *KCNJ2*	Drug-induced LQTS	Activator or opener of affected K^+ channel
		ND	AF	*KCNA5*	AF	
	Decreased L-type Ca^{2+} current	Action potential shortening	Combined SQTS and Brugada syndrome	*CACNA1C*, *CACNB2*	ND	Activator of peak Ca^{2+} current
	Decreased connexin channel current	ND	AF	*GJA5*	Some VT in ischaemic heart disease	Activation of gap junctions or increased gap junction conductance
	Decreased pacemaker current	Slowed spontaneous pacemaker rate	Sinus-node dysfunction	*HCN4*	Bradyarrhythmia	Activation of pacemaker current
Abnormal targeting of multiple Ca^{2+} control proteins	ND	DADs	AF, VF, bradycardia, type 4 LQTS	*ANK2*	Drug-induced LQTS	ND
Cardiomyopathic lesions, often associated with arrhythmias	Cytoskeletal-protein dysfunction	ND	Mostly dilated cardiomyopathy, heart block, AF, VF	*LMNA*, *DMD*, *DES*, *SGCD*, *ACTN2*, *VCL*, *CLP*, *LDB3*	Viral carditis	ND
	Sarcomere-protein dysfunction	EADs or DADs in some preparations	Mostly hypertrophic cardiomyopathy: myofibrillar disarray, non-concentric ventricular hypertrophy, AF, VT	*MYH7*, *MYBPC3*, *TNNT2*, *TNNI3*, *TPM1*, *MYL2*, *MYL3*, *ACTC1*, *TTN*	Acquired cardiac hypertrophy	
	Desmosome-protein dysfunction	ND	Arrhythmogenic right ventricular cardiomyopathy	*PKP2*, *DSP*, *JUP*, *DSG2*, *DSC2*, *DES*	ND	
	Metabolism and lysosomal protein dysfunction	Cytoplasmic aggregates due to defective lysosomes	Hypertrophic cardiomyopathy, ventricular pre-excitation	*LAMP2*, *PRKAG2*	Wolff–Parkinson–White syndrome	

The molecular, cellular and whole-heart phenotypes associated with fundamental molecular defects are indicated, together with the genetic and acquired arrhythmia syndromes that probably have the same pathophysiology. This information has led to specific interventions that are predicted or have been established to be antiarrhythmic. *ACTC1*, cardiac α-actin; *ACTN2*, α2-actinin; AF, atrial fibrillation; *ANK2*, ankyrin 2; AV, atrioventricular; *CACNA1C*, L-type Ca^{2+} channel, α1C subunit; *CACNB2*, L-type Ca^{2+} channel, β2-subunit; *CASQ2*, calsequestrin 2, cardiac; *CAV3*, caveolin 3; *CLP*, cardiac LIM protein; DAD, delayed afterdepolarization; *DES*, desmin; *DMD*, dystrophin; *DSC2*, desmocollin 2; *DSG2*, desmoglein 2; *DSP*, desmoplakin; EAD, early afterdepolarization; *GJA5*, gap-junction protein-α5 (also known as connexin 40); *HCN4*, hyperpolarization-activated cyclic-nucleotide-gated K^+ channel 4; *JUP*, junction plakoglobin (also known as β-catenin); *KCN*, K^+ channel; *LAMP2*, lysosomal-associated membrane protein 2; *LDB3*, LIM-domain-binding 3; *LMNA*, lamin A; LQTS, long-QT syndrome (QT-interval prolongation and 'torsades de pointes'-type polymorphic VT); *MYBPC3*, cardiac myosin-binding protein C; *MYH7*, β-myosin heavy chain; *MYL*, myosin light chain; ND, not determined; *PKP2*, plakophilin 2; *PRKAG2*, AMP-activated protein kinase-γ2; *SCGD*, δ-sarcoglycan; *SCN*, voltage-gated Na^+ channel; SR, sarcoplasmic reticulum; SQTS, short-QT syndrome (QT-interval shortening associated with AF and VF); *TNNI3*, cardiac troponin I; *TNNT2*, cardiac troponin T; *TPM1*, α-tropomyosin; *TTN*, titin; *VCL*, vinculin; VF, ventricular fibrillation; VT, ventricular tachycardia.

the presence of specific mutations has provided an impetus for the more widespread use of genetic testing.

Although relationships between mutations in disease-associated genes and clinical presentation can be seen when a large number of individuals are studied, it can be difficult to apply such data to individual patients and small families, because penetrance of the disease phenotypes caused by the mutations can vary substantially[37]. The mechanism underlying variable penetrance of these rather striking phenotypes is unknown, and environmental or genetic modifiers are thought to be involved. Strategies for moving forward in this area include very large studies of kindreds with variable penetrance of a single mutation, and studies in animal models using strains with differing genetic backgrounds.

Population genetics of arrhythmia susceptibility

Studies of large populations of patients who have atrial fibrillation[7,8] or had SCD[38–40] have identified family history as an important component of risk. Occasionally, common polymorphisms in the disease-associated genes listed in Table 1 have been reported to be modifiers of risk[41], and these are clear candidates for further study. In atrial fibrillation, and in particular lone atrial fibrillation, there is a high incidence of other family members being affected[7], further supporting a genetic aetiology. Linkage in such kindreds has allowed the identification of disease-associated loci[42,43], although no causative mutation has been identified at any of these loci so far. Another emerging approach is to carry out genome-wide association analysis in large cohorts who are at risk. This could be a special challenge in patients who had SCD, because their DNA is usually unavailable. Genome-wide association approaches have identified an atrial-fibrillation susceptibility locus near the *PITX2* gene, which encodes a transcription factor important for atrial myocardial development[44,45]. Similarly, variability in the normal QT interval has been reproducibly associated with polymorphisms in *NOS1AP*, which encodes a subunit of nitric-oxide synthase 1 (also known as nNOS)[46–48]. Before genome-wide association studies were possible, neither *NOS1AP* nor *PITX2* had been reported as a modulator of arrhythmia susceptibility. Thus, in addition to identifying disease-associated genes, such studies can also help to identify the signalling pathways in which the products of these genes are involved. Components of these pathways are thus candidate modulators for the arrhythmia susceptibility phenotype.

Why does contractile dysfunction promote arrhythmias?

The most powerful predictor of SCD in the general population is left ventricular contractile dysfunction, which is most often caused by acquired heart disease, such as mottled scarring due to infarction or hypertrophy due to hypertension. Contractile dysfunction in these cases is associated with obvious remodelling, including fibrosis and myofibrillar disarray (typical of hypertrophic cardiomyopathy); it is also associated with measurable proarrhythmic changes in fundamental electrophysiological properties, such as repolarization times and conduction velocity. However, although contractile dysfunction is linked to arrhythmia susceptibility, and although remodelling can create a proarrhythmic substrate, the actual mechanisms linking contractile dysfunction and arrhythmias remain only partly understood[49].

Genetic findings have pointed to one potential mechanism: 'leaky' ryanodine channels (also known as ryanodine receptors)[50]. These channels are multimolecular complexes that consist of both the channel itself — encoded by *RYR2* — and regulatory subunits, which release Ca^{2+} from sarcoplasmic-reticulum stores to initiate cardiomyocyte contraction (Fig. 2). Mutations in *RYR2* (ref. 51) and in *CASQ2*, which encodes the cardiac sarcoplasmic-reticulum luminal protein calsequestrin[52], have been associated with ventricular arrhythmias in humans, and the causality of this association has been confirmed by identifying sarcoplasmic-reticulum Ca^{2+} leak in mice engineered to carry these mutations[53–55]. These studies show that leaky channels result in cytosolic Ca^{2+} overload and, consequently, in delayed afterdepolarizations (DADs), which are presumed to arise from the activation of electrogenic sodium/calcium (Na^+/Ca^{2+}) ion exchange. In the mutant mice, arrhythmias are elicited by catecholamines and arise from trains of DADs or from DAD-mediated triggering of re-entry. This is consistent with ventricular tachycardia arising during adrenergic stimulation, the key clinical feature in humans who carry mutations in these genes.

Humans and mice with these mutations show surprisingly normal contractile function, considering the importance of Ca^{2+} release to cardiomyocyte contraction. However, leaky ryanodine channels have also been implicated in acquired heart failure associated with contractile dysfunction[56,57]. Some reports support the idea that adrenergic activation (a near-constant feature of heart failure) results in the hyperphosphorylation of ryanodine channels and renders them leaky, perhaps by altering their affinity for the key regulatory subunit calstabin 2 (also known as FKBP1B)[56,58,59]; it has therefore been suggested that inhibition of the leak can be antiarrhythmic[60]. However, more recent studies have not been able to confirm that ryanodine channels are hyperphosphorylated in heart failure[61–63].

Another mechanism by which contractile dysfunction might engender arrhythmia susceptibility was suggested by recent studies of hypertrophic cardiomyopathy. Arrhythmias associated with this disease have generally been attributed to micro-re-entry as a result of a characteristic myofibrillar disarray[64]. However, mutant myofilaments can directly alter cardiomyocyte Ca^{2+} homeostasis, for example by altering cytosolic Ca^{2+} buffering, with attendant arrhythmias arising even in the absence of marked myofibrillar disarray[65,66]. Similarly, arrhythmia susceptibility in the case of heart failure might be caused by a molecular lesion that can lead to both contractile dysfunction and arrhythmia, rather than by an indirect effect of proarrhythmic remodelling. One example is activation of Ca^{2+}/calmodulin-dependent kinase II (CAMKII)[67,68], which has been observed in a range of acquired heart diseases. In animal models of acquired heart disease, inhibition of CAMKII by small molecules or transgenes expressing inhibitory peptides can improve contractile function and prevent arrhythmias[69–73].

Thus, it is clear that abnormal function of Ca^{2+}-release channels or abnormal homeostasis of intracellular Ca^{2+} can both affect contractile function and cause ventricular arrhythmias. However, further studies are required to establish the underlying mechanisms in acquired heart disease, potentially providing new therapeutic targets.

Future directions

The study of fundamental mechanisms underlying arrhythmias has led to a marked improvement in treatment for some patients, such as those who can be cured with catheter ablation. Gene delivery and cell-based therapies are also being explored for treating the substrate for re-entry after myocardial infarction or for replacing electronic pacemakers with biological ones[74,75]. Studies of rare familial syndromes can identify molecules whose dysfunction leads to arrhythmias, ushering in an era of mechanism-based therapeutics. The great hope is that the same approach can be applied to common types of arrhythmia such as atrial fibrillation and SCD. Studies of large populations to identify common genetic variants that predispose individuals to arrhythmias hold similar promise for early detection and intervention in asymptomatic patients at high risk. Arrhythmias are an important public health challenge, and the opportunity to reinvent the therapeutic 'armamentarium' holds great potential to improve the outlook for patients. ■

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Stem-cell therapy for cardiac disease

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Heart failure is the leading cause of death worldwide, and current therapies only delay progression of the disease. Laboratory experiments and recent clinical trials suggest that cell-based therapies can improve cardiac function, and the implications of this for cardiac regeneration are causing great excitement. Bone-marrow-derived progenitor cells and other progenitor cells can differentiate into vascular cell types, restoring blood flow. More recently, resident cardiac stem cells have been shown to differentiate into multiple cell types present in the heart, including cardiac muscle cells, indicating that the heart is not terminally differentiated. These new findings have stimulated optimism that the progression of heart failure can be prevented or even reversed with cell-based therapy.

Ischaemic heart disease — characterized by reduced blood supply to the heart muscle — is the primary cause of death throughout the world, including most low-income and middle-income countries[1]. Obstruction of coronary arteries leads to myocardial infarction (heart attack) with the associated death of cardiomyocytes. This overloads the surviving myocardium and eventually leads to heart failure (see page 919). Other causes of heart failure, including chronic high blood pressure, are also characterized by a gradual loss of cardiomyocytes[2], and experimental inhibition of programmed cell death can improve cardiac function[3]. The only standard therapy for heart failure that addresses the fundamental problem of cardiomyocyte loss is cardiac transplantation. New discoveries on the regenerative potential of stem cells and progenitor cells for treating and preventing heart failure have transformed experimental research and led to an explosion in clinical investigation. The crucial point at which it is decided that laboratory evidence sufficiently supports clinical experimentation is particularly controversial in stem-cell therapy for heart failure, so it is timely to consider the current state of this field. In this review, we discuss the current knowledge of regeneration in the adult mammalian heart. We also consider the various stem-cell and progenitor-cell types that might regenerate the myocardium and review the major challenges to such therapy.

Cardiac regeneration

Few questions in cardiac regeneration are definitively resolved. But it is widely agreed that the regenerative capacity of human myocardium is grossly inadequate to compensate for the severe loss of heart muscle presented by catastrophic myocardial infarction or other myocardial diseases. By contrast, skeletal muscle in mammals can regenerate efficiently, even after widespread injury[4,5]. Satellite cells and other types of myoblast reside in skeletal muscle and form large numbers of new myotubes within days of muscle injury. However, a regenerative response does occur in the hearts of some vertebrates, such as zebrafish and newts, after injury[6,7]. In the normal state, newt cardiomyocytes, like those of mice and humans, rarely divide. But after a substantial injury, remaining cardiomyocytes initiate DNA synthesis and re-enter the cell cycle[6]. Division of existing cardiomyocytes seems to be the most important factor for cardiac regeneration in this animal. Dedifferentiation of cardiomyocytes near the injured zone occurs before their proliferation and is characterized by loss of expression of cardiac contractile proteins such as α-myosin heavy chain and troponin T (ref. 6). Cardiac regeneration in zebrafish might be initiated predominantly by undifferentiated stem or progenitor cells from the outer (epicardial) layer of the heart[8]. Further study of newts and zebrafish will define more clearly whether cardiac regeneration in these organisms requires dedifferentiation, proliferation and subsequent differentiation of existing cardiomyocytes, or whether regeneration is driven by the recruitment of stem cells to the injured site. By contrast, in mammalian hearts cardiomyocytes bordering a myocardial infarction rarely divide after injury[9,10], although transgenic overexpression of specific genes in mice can increase cardiomyocyte division[10].

There is strong evidence that endothelial cells are renewed by bone-marrow-derived progenitor cells[11], but the idea that cardiomyocytes are renewed by such cells is vigorously debated[11]. Less controversially, many laboratories have now demonstrated that adult mammalian myocardium has a population of resident cardiac stem cells (CSCs) with the potential to differentiate into cardiomyocytes and other cell types such as endothelial and vascular smooth muscle cells[12–15]. It has been proposed that CSCs support basal turnover of cardiomyocytes[6,11], but this probably occurs at a very low rate in the absence of injury[16]. CSCs have a high proliferation and differentiation potential *in vitro*[12–15], and the possibilities of expanding autologous CSCs *ex vivo* or stimulating the regeneration capacity of these cells *in vivo* are exciting options for therapeutic regeneration.

The realization that regenerative mechanisms do exist in mammalian myocardium brings into sharp focus the problem of defining the barriers that could be preventing regeneration, including the ischaemia, inflammation and fibrosis that characterize various stages of infarcted myocardium (Fig. 1). This hostile microenvironment might prevent the activation of resident CSCs and thus also reduce the success of exogenous cell therapies. Some components of the inflammatory response might be essential for promoting angiogenesis and progenitor-cell recruitment, but excessive inflammation might also prevent the recruitment and survival of progenitor cells. Similarly, some degree of fibrosis is required to prevent myocardial rupture after a myocardial infarction, but dense fibrosis presents a formidable physical barrier to regenerating cells[17]. It is likely that no single factor defines the hostile microenvironment of injured myocardium. In the MRL mouse strain, multiple genetic loci contribute to increased regenerative capacity in some organs, such as faster wound closure with minimal scar formation[18]. Several beneficial mechanisms

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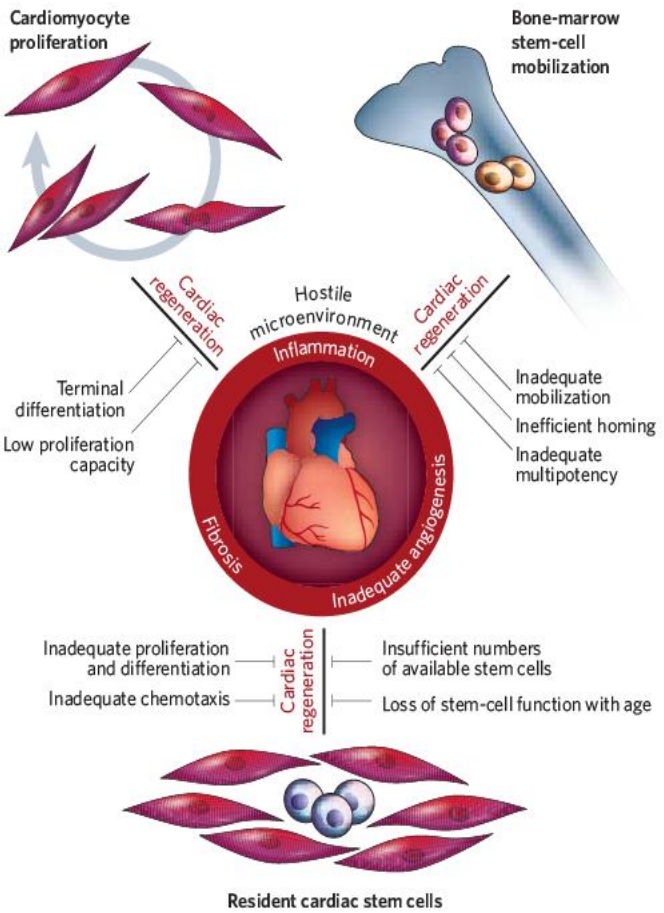

Figure 1 | Mechanisms of, and potential barriers to, endogenous cardiac regeneration. Cardiomyocyte proliferation has a role in the regeneration of the heart in some vertebrates, but the proliferative capacity of these cells is limited in the adult mammalian heart. After injection or mobilization of bone-marrow-derived cells, bone-marrow-derived cardiomyocytes have been detected at very low rates in adult hearts, indicating that bone-marrow-derived progenitor cells may be able to migrate to the heart and differentiate into cardiomyocytes or fuse with existing cardiomyocytes. However, these findings are still controversial after half a decade of intensive research. Resident cardiac stem cells (CSCs) with the potential to differentiate into multiple myocardial cell types, including cardiomyocytes, have been isolated from myocardium. It is not completely clear what barriers prevent endogenous CSCs from regenerating myocardium more effectively.

working together seem to contribute to the improved cardiac regenerative capacity sometimes observed in these mice, including increased vascularization and cell proliferation as well as reduced apoptosis and fibrosis[19].

Which stem cells should be used for cardiac therapy?

Perhaps the most stunning aspect of current progress towards cardiac regeneration is the wide variety of cell types that have been considered as candidates for therapeutic delivery in humans (Fig. 2). This myriad of cell types reflects the unmet medical need for treating heart disease, and hence the large amount of experimental effort being put into devising cell-based therapies. It also points to the lack of mechanistic understanding at many levels. The ideal cell type has not yet emerged, and few studies have directly compared different stem-cell types[20].

Skeletal myoblasts

One of the first cell-based cardiac regeneration strategies was injection of autologous skeletal myoblasts into ischaemic myocardium[21]. Myoblasts are resistant to ischaemia, can differentiate into myotubes *in vivo* (but not into cardiomyocytes[11]) and improve ventricular function in laboratory animal experiments[21]. Myotubes do not integrate electrically with surviving cardiomyocytes[11] and thus do not beat in synchrony with the surrounding myocardium. Human trials of myoblasts in heart failure are ongoing; however, some have been terminated because of lack of efficacy[22], and it is unlikely that skeletal myoblasts will be able to truly regenerate myocardium. Mouse skeletal muscle contains a population of non-satellite cells that can differentiate into spontaneously beating cells with cardiomyocyte features[23], but an equivalent population of cardiac-committed cells in human skeletal muscle has not yet been described.

Bone-marrow-derived cells

A subset of bone-marrow-derived haematopoietic cells were the first adult stem cells or progenitor cells reported to differentiate into cardiomyocytes when transplanted into infarcted hearts of mice[24]. The first evidence that adult bone-marrow-derived progenitor cells participate in the formation of cardiomyocytes in adult human hearts was based on reports of Y-chromosome-positive cardiomyocytes in female donor hearts transplanted in male recipients[25]. Animal studies of bone-marrow transplantation with labelled haematopoietic stem cells followed by myocardial infarction revealed cardiomyocytes derived from the transplanted cells, but at an exceptionally low rate[26]. However, other studies in animals have not demonstrated differentiation of haematopoietic progenitor cells into cardiomyocytes[24,27,28] or improvement in cardiac function[29]. Currently, no consensus exists on whether bone-marrow-derived progenitor cells differentiate into cardiomyocytes *in vivo*.

Endothelial progenitor cells (EPCs) are a subset of haematopoietic cells found in the bone marrow that have the potential to differentiate into endothelial cells[30]. EPCs have not been shown to differentiate into cardiomyocytes *in vivo*, but they probably have a role in promoting angiogenesis[10,30]. In addition to directly contributing to the vasculature required to deliver nutrients to new cardiomyocytes, endothelial cells can also provide paracrine survival signals to cardiomyocytes[31]. EPCs are readily isolated from the blood and the bone marrow, and clinical studies suggest that cell-based therapy with EPCs can improve myocardial function[32]. But definitions of EPCs vary such that different studies probably use different types of cell, making comparisons difficult.

So far, most clinical studies have used bone-marrow mononuclear cells and showed either no benefit or small (but possibly clinically important) improvements in cardiac function[32]. The mechanisms of these functional improvements are unknown, but it is unlikely that the improvements result from differentiation of the injected cells into cardiomyocytes. Growth factor and cytokine release by injected cells is frequently suggested as a potential mechanism of action[32], and improved microvascular function has been shown in the REPAIR-AMI study[33].

The bone marrow also contains mesenchymal stem cells, multipotent cells that can differentiate into osteoblasts, chondrocytes and adipocytes[34]. A subset of mesenchymal stem cells can differentiate into cardiomyocytes under specific conditions *in vitro*[20,34]. Differentiation into cardiomyocytes *in vivo* has also been observed, but at an extremely low rate[35,36]. A potential advantage of mesenchymal stem cells is that they are less immunogenic than other stem cells, potentially allowing allogeneic cell therapy[36]. Mesenchymal stem cells can provide paracrine growth factor support for other cells present in injured myocardium[34,37], and this could be the major mechanism for the beneficial effects of these cells. To increase the therapeutic potency of mesenchymal stem cells, they have been genetically modified to overexpress prosurvival factors, angiogenic factors, growth factors, or stem-cell homing factors[34]. A cautionary note, however, was sounded by a report that found these cells differentiating into bone-forming osteoblasts — instead of cardiomyocytes — in transplanted mouse hearts[38]. These results highlight the principle that even if multipotent stem cells, such as mesenchymal stem cells, have cardiomyocyte differentiation potential, preventing differentiation into other cell types is a crucial consideration. In addition to EPCs and mesenchymal stem cells, the bone marrow contains other populations of putative progenitor or stem cells with the potential to differentiate into myocytes[39].

Embryonic stem cells

Embryonic stem (ES) cells are the prototypical stem cells. They unambiguously fulfil all requirements of stem cells: clonality, self renewal and

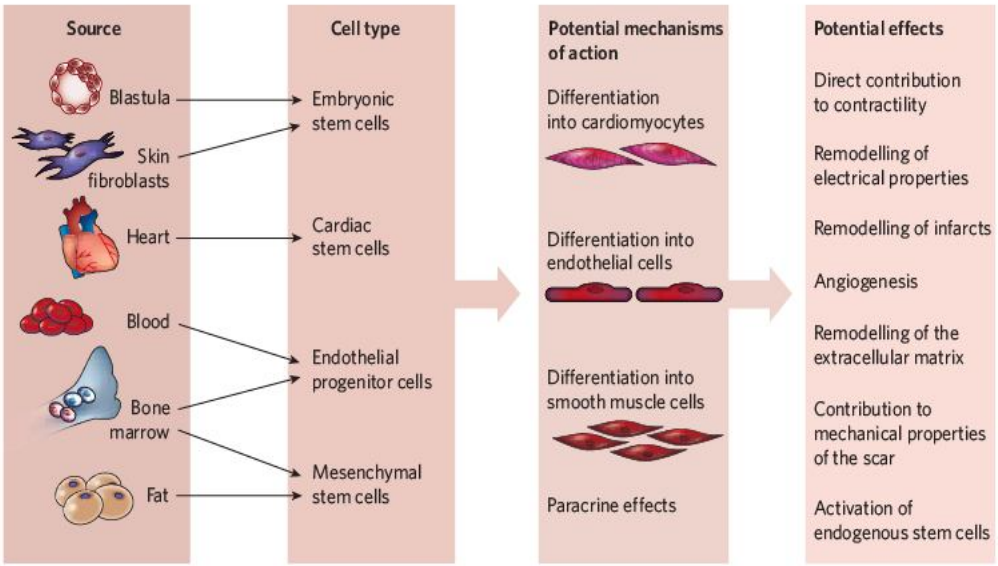

Figure 2 | Many cell types and mechanisms have been proposed for cardiac therapy. Stem cells and progenitor cells can be isolated from either autologous or allogeneic sources. Different types of stem cell and progenitor cell have been shown to improve cardiac function through various mechanisms, including the formation of new myocytes, endothelial cells and vascular smooth muscle cells, as well as through paracrine effects.

multipotentiality[40]. ES cells can differentiate into any cell present in the adult organism and have the potential to completely regenerate the myocardium. Two of the obstacles that stand in the way of the therapeutic use of ES cells are immunological rejection and the propensity of ES cells to form teratomas when injected *in vivo*[11,41]. As knowledge of pathways for ES-cell differentiation and for heart embryonic development increases, ES-cell differentiation might become more controllable. Methods to limit teratoma formation include genetic selection of differentiated ES cells[42], or differentiation of ES cells *in vitro* into cardiomyocytes or endothelial cells before injection[43,44]; for example, tumour-necrosis factor promotes the differentiation of ES cells into cardiomyocytes[45]. Differentiated ES cells can survive and improve myocardial function if delivered to the myocardium in a rich prosurvival cocktail[43]. An inherent difficulty in controlling the growth and differentiation of ES cells and other pluripotent stem cells is that the timing with which specific signalling pathways are activated might be crucial. For example, recent studies on mouse and zebrafish embryos reveal that the role of the Wnt–β-catenin pathway in cardiac development varies depending on the developmental stage[46].

Endogenous cardiac stem cells

Because allogeneic cells face immunological challenges that would probably require immunosuppression, the isolation of endogenous adult mammalian CSCs on the basis of cell-surface markers has generated great enthusiasm[47]. However, a definitive marker for CSCs has not yet been identified. Mammalian myocardium includes a small proportion of stem cells that express the cell-surface markers Kit[12] or Sca1 (ref. 48). Side-population cells, identified by their ability to exclude Hoechst dye, were first described in the bone marrow as being enriched in haematopoietic stem cells, but they are also found in other organs, including the heart[14]. Some side-population cells express Kit and/or Sca1, and like Kit^+ CSCs and $Sca1^+$ CSCs, side-population cells can generate cardiomyocytes *in vitro* and *in vivo*[49]. In addition to Kit^+ CSCs, $Sca1^+$ CSCs and side-population cells, a fourth population of CSCs expresses the transcription factor Isl1 (ref. 13). Lineage-tracing experiments have shown that Isl1-expressing cells can differentiate into endothelial, endocardial, smooth muscle, conduction system, right ventricular and atrial myogenic lineages during the development of the embryonic heart[50]. Isl1-expressing cells are also present in the adult mammalian heart, but they are limited to the right atrium, are found in smaller numbers than in embryonic hearts[13] and have an unknown physiological role. Recently, epicardium-derived progenitor cells have been described that show angiogenic potential[51,52].

CSCs can be isolated and expanded from human myocardial samples obtained using a minimally invasive biopsy procedure[53,54]. Thus, from autologous CSCs, it might be possible to generate enough cells to transplant into patients with heart failure, a procedure that would have minimal risk of immune rejection or teratoma formation. But no clinical data using CSCs are available yet, and many important questions about CSCs remain unanswered. Can their *in vitro* proliferative and differentiation potential translate to long-term *in vivo* function? Do they retain their cardiogenic potential in disease states, or with advanced

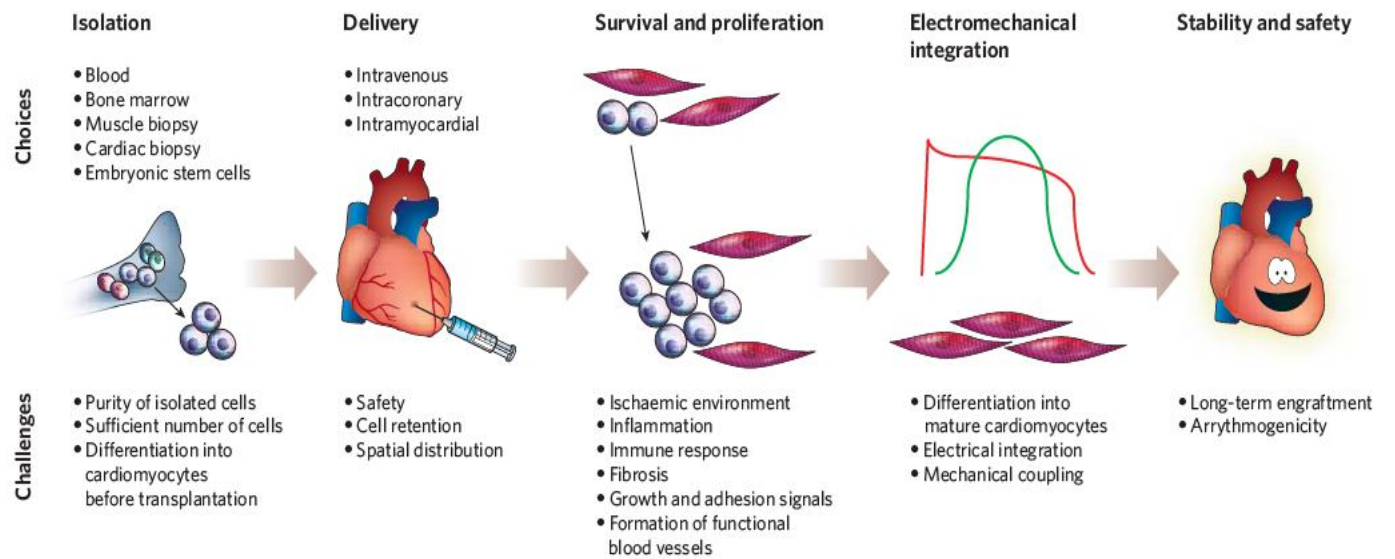

Figure 3 | Challenges to stem-cell therapy for cardiac disease. True cardiac regeneration with stem-cell therapy will require careful consideration at each step, from isolation of the cells to their stable and safe long-term integration.

Box 1 | Fundamental questions in CSC therapy

Is the optimal opportunity for CSC therapy early after myocardial infarction or in chronic cardiomyopathy?

- The advantages of therapy immediately after myocardial infarction include preservation of myocardial architecture. Fibrosis is minimal and cardiac remodelling of the remaining viable myocardium has not yet occurred. Paracrine mechanisms that promote cell survival or promote angiogenesis might be a good strategy for this early period.
- The advantages of therapy in chronic cardiomyopathy include clinical stability and a larger target patient population. The stable scar tissue has less intense inflammation. Contractile cells or cells that can differentiate into contractile cells might be the best choice in this situation.

Can allogeneic cells be used?

- Allogeneic cells that are 'off the shelf' can be generated with a high degree of homogeneity and quality control. Therapy immediately after myocardial infarction is possible at a potentially lower cost.
- Allogeneic cells will be more prone to rejection than autologous cells, and the need for chronic immunosuppression could increase the risk over the benefit.

How much mechanistic understanding is needed before clinical trials?

- The fundamental mechanisms of cardiac regeneration in humans are incompletely defined, and the degree of experimental evidence needed to justify clinical trials is controversial.
- Although current enthusiasm for translating CSC therapy into humans seems appropriate, patient safety and clinical trials that adequately test defined hypotheses are key considerations.
- At this early stage, clinical trials should maximize the potential for obtaining rigorous mechanistic information.

What are the primary mechanism by which different types of stem cell or progenitor cell improve myocardial performance?

- Different cell types might be capable of improving cardiac function by distinct mechanisms.
- ES cells and resident CSCs might have true mechanical potential, as they can differentiate into mature cardiomyocytes.
- Mesenchymal stem cells might improve angiogenesis by paracrine factors, but other mechanisms are plausible.
- EPCs have the greatest potential for angiogenesis.

Will cells be necessary?

- The greatest potential of stem-cell therapy is true cardiac regeneration through differentiation into cardiomyocytes. However, studies so far suggest that the differentiation of bone-marrow progenitor cells into cardiomyocytes is a rare event, and the positive effects of cell-based therapy could be due to cell-derived paracrine factors.
- Transplantation of stem cells or progenitor cells for their paracrine effects remains a reasonable strategy because the beneficial paracrine factors remain unidentified and because multiple factors might be functioning synergistically.
- If paracrine cell-derived factors that improve cardiac function are identified, then protein-based therapy might be more easily translated into clinical benefits than cell-based therapy.

age? To what extent are the different types of CSC that have been identified really different cell types? Or are they the same cell type at varying stages of differentiation[47]? Are all the CSCs that have been isolated so far truly capable of self renewal *in vivo*, and is their plasticity real or just a laboratory artefact?

The identification of various cell types with cardiogenic potential has also stimulated interest in improving cardiac function by mobilizing endogenous stem cells and progenitor cells without removing and expanding the cells *ex vivo*. Proangiogenic factors either produced by ischaemic tissues or administered exogenously can mobilize EPCs and improve vascular function[47,55]. In addition to mobilizing stem cells from the bone marrow, homing signals can be important for guiding stem cells to the injured myocardium. For example, local myocardial delivery of the chemoattractant cytokine CXCL12 can improve homing of EPCs to the heart[56]. Furthermore, mesenchymal stem cells can be found in small numbers in peripheral blood[57], indicating that non-haematopoietic stem cells or progenitor cells circulate and might have the capability to home to injured tissues. Like stem cells or progenitor cells in other tissues, CSCs reside in clusters consistent with the existence of cardiac niches[53]. The factors that attract these cells out of their putative niches to an injury site remain to be defined. The question also remains whether CSCs stably reside in the heart or are derived from other tissues such as the bone marrow, as has been suggested for Kit^+ cells[58].

Strategies and challenges

Some of the challenges facing stem-cell therapy for cardiac disease are outlined in Fig. 3. The most obvious question to be answered by preclinical studies is which type of stem cell or progenitor cell is the best candidate for therapy[20]. Bone-marrow-derived progenitor cell therapy has thus far proven safe and beneficial under defined circumstances (acute myocardial infarction), but the cells' regeneration potential is controversial. CSCs have the potential to be patient specific, but isolation and culture procedures are in the early development stages[53,54]. ES cells have the greatest differentiation potential, but face ethical barriers and also have the greatest risk for teratoma formation[41]. Whether ES-cell derivatives will be rejected by the host immune response is still under debate; in principle, however, rejection can be avoided by using cells from a bank of only 150 donors with different HLA types[59]. If the new technology that reprogrammes human and mouse fibroblasts to ES-like cells can be harnessed[60–63], the use of patient-specific reprogrammed cells could reduce or even eliminate immune rejection. A crucial issue in designing more rational cell-based therapy approaches for cardiac disease is understanding the mechanisms by which each of the stem-cell or progenitor-cell types can affect myocardial performance[11,55] (Fig. 2 and Box 1). Also, different cardiac pathologies — for example, acute myocardial infarction and chronic ischaemic cardiomyopathy — might require different types of stem cell or progenitor cell (Box 1).

Another issue for cell-based therapy is determining the optimal route of delivery. Cells can be injected intravenously, into coronary arteries or directly into the myocardium. Myocardial stem-cell and progenitor-cell delivery with these approaches in numerous phase I clinical trials has thus far revealed few serious adverse effects[32]. Recent trials that include a control group are listed in Table 1. Retention of cells immediately after delivery is highly dependent on the delivery strategy: if cells are injected intramyocardially during open-chest surgery, many cells are lost through the vasculature[21], and few cells infused into coronary arteries ultimately engraft[20]. Survival in the inflammatory environment of infarcted myocardium is a challenge common to all types of transplanted cell, as typically 90% of the cells die within a week[11]. A sufficiently large cell graft with appropriate structural and functional properties will be needed. Because persistent ischaemia also limits cell survival, revascularization and improving angiogenesis could be essential components of cell-based therapy. Survival and integration of transplanted cells can also be improved by embedding them in matrices such as collagen[21] or Matrigel[43], by implanting cells as monolayer sheets[35] or by simultaneously delivering growth factors[43,64]. Long-term electromechanical stability and appropriate structural and functional electromechanical integration[65] with host tissue will be essential for cardiac regeneration.

Future directions

In many studies, the number of differentiated and functionally integrated myocytes derived from transplanted stem cells is too small to explain the observed improvements in cardiac function[37,47]. Improved cardiac function in these studies might be driven by paracrine mechanisms, and identification of these cell-derived paracrine factors could lead to effective therapies without delivery of the cells themselves. Local intramyocardial delivery of cytokines or growth factors could be more

Table 1 | Overview of clinical trials of stem-cell or progenitor-cell delivery to the heart

Cell type	Study design	Number of patients*	Mean follow-up duration (months)	Number of cells injected	Route of injection	Ejection fraction versus control (%)†	Source‡
BMMNC	R-SB	60	12	10^8	Intracoronary	+7.0 ($P=0.03$)	Meluzin *et al.*[67] (2007)
	R-SB	51	3	2×10^8	Intracoronary	+4.1 ($P=0.001$)	Assmus *et al.*[32] (2006)
	R-SB	66	3	10^8	Intracoronary	+3 ($P=0.04$)	Meluzin *et al.*[68] (2006)
	R-SB	204	12	2.4×10^8	Intracoronary	Decreased mortality	Schächinger *et al.*[69] (2006)
	R-SB	20	6	4×10^7	Intracoronary	+6.7 (NS)	Ge *et al.*[32] (2006)
	R-SB	20	4	6×10^7	TEIM	+2.5 (NS)	Hendrikx *et al.*[32] (2006)
	R-DB	67	4	1.7×10^8	Intracoronary	+1.2 (NS)	Janssens *et al.*[32] (2006)
	R-SB	100	6	8.7×10^7	Intracoronary	-3.0 ($P=0.05$)	Lunde *et al.*[32] (2006)
	R-SB	60	18	2.5×10^9	Intracoronary	+2.8 (NS)	Meyer *et al.*[32] (2006)
	Cohort§	36	3	3×10^8	TEIM	+4.0 (NS)	Mocini *et al.*[32] (2006)
	R-SB	204	4	2.4×10^8	Intracoronary	+2.5 ($P=0.01$)	Schächinger *et al.*[32] (2006)
	Cohort§	36	3	9×10^7	Intracoronary	+7.0 ($P=0.02$)	Strauer *et al.*[32] (2005)
	Cohort§	20	12	2.6×10^7	TEIM	+8.1 (NS)	Perin *et al.*[32] (2004)
	Cohort§	20	3	2.8×10^7	Intracoronary	+1.0 (NS)	Strauer *et al.*[32] (2002)
CPC	Cohort§	54	6	5×10^9	Intracoronary	+6.0 ($P=0.04$)	Tatsumi *et al.*[70] (2007)
	Cohort§	73	6	2×10^9	Intracoronary	+2.8 (NS)	Choi *et al.*[71] (2007)
	R-SB	47	3	2×10^7	Intracoronary	+0.8 (NS)	Assmus *et al.*[32] (2006)
	R	82	6	1.4×10^9	Intracoronary	-0.2 (NS)	Kang *et al.*[32] (2006)
	Cohort§	70	6	7.3×10^7	Intracoronary	+5.5 ($P=0.04$)	Li *et al.*[32] (2006)
	SB	26	3	7×10^7	Intracoronary	+7.2 (NS)	Erbs *et al.*[32] (2005)
$CD133^+$	Cohort§	27	6	NA	Intramyocardial ||	NA	Ahmadi *et al.*[72] (2007)
	Cohort§	55	6	6×10^6	Intramyocardial ||	+6.3 ($P=0.02$)	Stamm *et al.*[73] (2007)
	Cohort§	35	4	1.3×10^7	Intracoronary	+2.8 (NS)	Bartunek *et al.*[32] (2005)
$CD34^+$	R-DB	24	6	3.5×10^7	TEIM	NA	Losordo *et al.*[74] (2007)
SMB	R-DB	97	6	NA	Intramyocardial ||	+3 ($P<0.04$)	MAGIC[22] (2007)
	Cohort§	26	12	2.5×10^8	Intramyocardial ||	+14.5 ($P<0.01$)	Gavira *et al.*[75] (2006)
	Cohort§	12	12	2.1×10^8	TEIM	+11.6 ($P<0.05$)	Ince *et al.*[76] (2004)
MSC	R	48	12	5×10^6	Intracoronary	-3 (NS)	Chen *et al.*[77] (2006)
	R-SB	69	6	6×10^{10}	Intracoronary	+12.0 ($P=0.01$)	Chen *et al.*[32] (2004)
MSC + EPC	Cohort§	22	4	3×10^6	Intracoronary	+0.3 (NS)	Katritsis *et al.*[32] (2005)
BMC	R-DB	20	6	NA	Intracoronary	+9.2 ($P<0.05$)	Ruan *et al.*[32] (2005)

BMC, bone-marrow-derived cells (unspecified); BMMNC, bone-marrow mononuclear cell; CPC, circulating progenitor cell; DB, double blinded; EPC, endothelial progenitor cell; MSC, mesenchymal stem cell; NA, not available; NS, not significant; R, randomized; SB, single blinded; SMB, skeletal myoblast; TEIM, transendocardial intramyocardial injection. *The number of patients is the sum of individuals in the control and treatment groups; almost all studies have equal numbers in each group. †Ejection fraction is the proportion of blood in the left ventricle that is ejected into the aorta during each heartbeat; this is a measure of cardiac function. ‡The author names refer to the original report, and the reference number cited indicates either the original report or a meta-analysis (or review) in which the original report is discussed. §Cohort denotes a non-randomized and non-blinded study. ||Intramyocardial indicates injection through the epicardial side of the heart.

reproducible than injection of heterogeneous populations of stem cells or progenitor cells. Paracrine signalling derived from non-surviving cells might not provide true myocardial regeneration, but new therapies for heart failure are so badly needed that the opportunity to try to exploit this possibility cannot be passed up. For example, one strategy might be delivery of proteins such as periostin, which has been shown to induce proliferation of cardiomyocytes after myocardial infarction[66].

Until now, clinical trials have used cell types that are readily available (bone-marrow mononuclear cells and EPCs), but these cell types do not necessary reflect stem-cell populations that are most likely to regenerate myocardium. Achieving the longer-term goal of true cardiac regeneration will probably require more than simply injecting the right type of cells in the right place. Understanding cardiomyocyte development and turnover — both in normal development and after injury — will be essential for guiding the development of stem-cell-based therapies. Defining the factors present in the hostile microenvironment of injured myocardium that limit the survival and functional integration of transplanted cells is also crucial. As the barriers that prevent human cardiac regeneration are further defined, clinical trials should proceed with caution and with a paramount concern for patient safety. ■

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The developmental genetics of congenital heart disease

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Congenital heart disease is the leading cause of infant morbidity in the Western world, but only in the past ten years has its aetiology been understood. Recent studies have uncovered the genetic basis for some common forms of the disease and provide new insight into how the heart develops and how dysregulation of heart development leads to disease.

Congenital heart disease usually refers to abnormalities in the heart's structure or function that arise before birth. They occur often and in many forms. Congenital heart diseases are found in 19–75 of every 1,000 live births, depending on which types of defect are included[1], and the incidence is higher if fetuses that do not survive to term are included[2]. This number excludes cardiomyopathies, conduction-system disease and laterality defects, which, although inherited and present at birth, are considered separately because of their distinct clinical presentation. A clear picture of how the heart forms is crucial for understanding the genesis of congenital heart disease, because dysregulation of heart development is at the root of the disease. This review focuses on genetic studies over the past ten years that have pinpointed the causes of inherited congenital heart diseases. Together with recent insight into how the heart normally develops, these studies have considerably improved the understanding of congenital heart diseases.

The clinical picture

Congenital heart diseases affect most parts of the heart (Fig. 1) and can be classified into three broad categories: cyanotic heart disease, left-sided obstruction defects and septation defects. Infants with cyanotic heart disease appear blue as a result of the mixing of oxygenated and deoxygenated blood. Defects that can contribute to this condition include transposition of the great arteries (TGA), tetralogy of Fallot (TOF), tricuspid atresia, pulmonary atresia, Ebstein's anomaly of the tricuspid valve, double outlet right ventricle (DORV), persistent truncus arteriosus (PTA) and total anomalous pulmonary venous connection. Left-sided obstructive lesions, the second main type of congenital heart disease, include hypoplastic left heart syndrome (HLHS), mitral stenosis, aortic stenosis, aortic coarctation and interrupted aortic arch (IAA). Septation defects, the third main type of congenital heart disease, can affect septation of the atria (atrial septation defects, ASDs), septation of the ventricles (ventricular septal defects, VSDs) or formation of structures in the central part of the heart (atrioventricular septal defects, AVSDs). Other types of congenital defect that do not fit neatly into the three main categories are bicuspid aortic valve (BAV) and patent ductus arteriosus (PDA). The most common congenital heart disease is BAV, and septation defects are the next most common.

Mortality and morbidity vary with the severity of the congenital heart disease and can be serious. The multiple surgeries needed to correct many of the anatomical defects can be debilitating, and quality of life is often greatly compromised. Children with congenital heart disease frequently develop neurological disorders, even if the child has not undergone surgery, indicating an important secondary effect of congenital heart diseases *in utero*[3]. It is therefore crucial to understand the effects of congenital heart diseases on prenatal and postnatal physiology.

Although the major underlying defects that cause congenital heart disease are thought to be mutations in regulators of heart development during embryogenesis[4], epidemiological data also point to environmental influences[5]. For example, prenatal exposure to angiotensin-converting-enzyme inhibitors increases the risk of several congenital malformations, including those that cause heart diseases[6]. However, these epidemiological studies have mostly suggested risk rather than pinpointing the underlying disease mechanisms.

A genetic component for congenital heart diseases was initially implicated by their recurrence in families, and by studies showing an association of congenital heart diseases with inherited microdeletion syndromes, in which a chromosomal region containing many genes is deleted. But until ten years ago, aside from these microdeletion syndromes, little was known about the genetics of congenital heart diseases. Indeed, geneticists and clinicians debated whether congenital heart diseases could be caused by a single-gene defect. Confounding these discussions were cases in which different members of one family might have anatomically distinct defects — for example, one member with an ASD, one with TOF and one with a VSD. These apparently discordant clinical phenotypes arising within one family were difficult to rationalize. In addition, mild or intermediate ('forme-fruste') defects, such as atrial septal aneurysms, are sometimes either discounted or not diagnosed, and thus the pattern of genetic inheritance of congenital heart diseases is often not clear.

New concepts in heart development

Congenital heart diseases arise from abnormal heart development during embryogenesis, so understanding how the heart forms normally is important (Fig. 2). The regulatory mechanisms involved in establishing the early heart and regulating its morphogenesis have been studied extensively[7,8]. The earliest cardiac progenitors arise from lateral plate mesoderm, controlled by a cascade of interacting transcription factors. Additional inputs come from secreted molecules, such as fibroblast growth factors, bone morphogenetic proteins, Wnt proteins and others[8].

Recent findings have clarified the origin of cardiac precursors and their regulation. Discovery of a 'second' heart field (SHF) led to a rethinking of the origin and patterning of the embryonic heart[9]. The SHF is medial and dorsal to the early differentiating cardiomyocytes that comprise the 'cardiac crescent', and gives rise to a large portion of the heart, including the outflow tract, right ventricle and most of the atria (Fig. 2a). The SHF is further subdivided into a number of lineage

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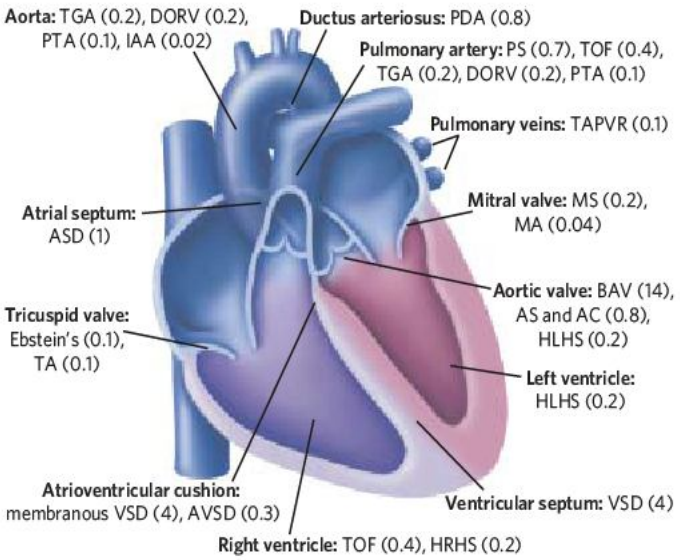

Figure 1 | Congenital heart defects. This diagram of the adult heart illustrates the structures that are affected by congenital heart diseases, with the estimated incidence of each disease per 1,000 live births indicated in parentheses. AC, aortic coarctation; AS, aortic stenosis; ASD, atrial septal defect; AVSD, atrioventricular septal defect; BAV, bicuspid aortic valve; DORV, double outlet right ventricle; Ebstein's, Ebstein's anomaly of the tricuspid valve; HLHS, hypoplastic left heart syndrome; HRHS, hypoplastic right heart; IAA, interrupted aortic arch; MA, mitral atresia; MS, mitral stenosis; PDA, patent ductus arteriosus; PS, pulmonary artery stenosis; PTA, persistent truncus arteriosus; TA, tricuspid atresia; TAPVR, total anomalous pulmonary venous return; TGA, transposition of the great arteries; TOF, tetralogy of Fallot; VSD, ventricular septal defect. (Image courtesy of F. Yeung, University of Toronto, Canada.)

pools[9], which contribute either to anterior structures (such as the outflow tract) or posterior components (such as the atria). These findings help explain how mutations associated with congenital heart disease can, by affecting only specific cell lineages within the SHF, result in defects in specific heart structures.

Progress has also been made in understanding how the pool of undifferentiated cardiac precursors that contribute to the SHF arises and how their further development is regulated. Intriguingly, the cardiovascular lineages — myocardial, endocardial and smooth muscle — all derive from common precursors that sequentially branch off as specialized cell types[10–12]. This strategy is similar to that used by the haematopoietic system. Regulation of the expansion and allocation of the early heart precursors has been attributed, in large part, to the Wnt family of secreted molecules[13]. However, which Wnts are important and where they signal from have yet to be determined.

An important principle in heart development is that regulation of different cell lineages must be tightly controlled so that the correct lineage differentiates at the correct time and in the correct location. Recent work in zebrafish has shown that a key level of regulation might be the active repression of the cardiac programme in anterior lateral plate mesoderm adjacent to heart precursors, by imposition of a haematopoietic and endocardial programme[14]. Heart-field size in zebrafish is negatively controlled by retinoic acid[15] and is thus influenced by both cell-type-specific determinants and broad patterning cues. In the SHF in mice, the transcription factor NKX2-5 limits the expansion of cardiac progenitors and promotes their differentiation potential: in mice lacking NKX2-5, early overproduction of progenitor cells is followed by impaired proliferation of SHF cells, resulting in a smaller outflow tract and right ventricle[16].

The role of transcription factors in heart development is well established[7,8], but less is known about the role of factors that modify the structure of chromatin; that is, the fibres of DNA and proteins (known as histones) that make up chromosomes and whose packaging can restrict or allow gene activation. BAF60C (also known as SMARCD3), a subunit of the Swi/Snf-like chromatin-remodelling complex BAF, physically links cardiac transcription factors to the BAF complex. Loss of BAF60C results in severe defects in cardiac morphogenesis and impaired activation of a subset of cardiac genes[17]. Interestingly, a partial reduction in BAF60C levels leads to more-restricted defects in outflow tract formation, suggesting that regulation of the dosage of chromatin-remodelling complexes is crucial for normal heart development[17]. Whereas BAF complexes alter the structure of chromatin, other chromatin-remodelling proteins modify histones, and these proteins are also important for heart formation. The muscle-restricted histone methyltransferase SMYD1 (also known as BOP) is a crucial regulator of cardiac chamber growth and differentiation[18]. With regard to the heart, histone deacetylases have mostly been characterized as having a role in hypertrophy, but they are also important in heart development[19].

Transcription-factor interactions

Human genetic studies have identified numerous genes that are responsible for inherited and sporadic congenital heart diseases. Most of these genes encode transcription factors that regulate specific events in heart development, such as ventricular septation or outflow tract morphogenesis (Fig. 3). The first identified single-gene mutation giving rise to an inherited congenital heart disease was in the T-box transcription factor gene *TBX5*, the causative gene in Holt–Oram syndrome (HOS)[20,21]. HOS predominantly includes ASDs, VSDs and conduction-system defects. Soon after this first discovery, mutations in *NKX2-5* were identified in families with inherited ASDs and atrioventricular block[22], and *NKX2-5* mutations were also found in families with diverse congenital heart-disease lesions, including VSDs, Ebstein's anomaly and TOF[23]. These results provided the insight that haploinsufficiency of a developmentally important transcription factor is at the root of disease and could explain the characteristic dominant pattern of disease inheritance. The importance of transcription-factor dosage was confirmed by using mouse models[24,25] (Fig. 4 and Box 1). An important finding from this work is that TBX5 and NKX2-5 interact physically and synergistically to activate their downstream targets[25,26], providing insight into how mutations altering either of these proteins affect cardiac gene expression and lead to disease.

The importance of interacting transcription factors was further emphasized by studies showing that mutations in the zinc-finger transcription-factor-encoding gene *GATA4* cause inherited septation defects[27]. GATA4, long studied as a regulator of cardiac gene expression, physically interacts with NKX2-5 (refs 7, 8). Defective interactions between GATA4 and NKX2-5, and between GATA4 and TBX5, might underlie congenital heart diseases caused by *GATA4* mutations. Thus, on the basis of positional cloning in three types of congenital heart disease with overlapping defects, three interacting cardiac transcription factors were identified as dosage-sensitive regulators of heart formation.

In mouse chromosome-engineering studies, another transcription factor gene, *Tbx1*, was pinpointed as the likely single-gene culprit in 22q11 microdeletion syndrome (also known as DiGeorge syndrome), which is characterized by congenital heart diseases such as TOF, PTA and IAA[28,29]. This conclusion was supported by the identification of *TBX1* missense mutations in patients with features of 22q11 microdeletion syndrome but without a microdeletion[30]. *Tbx1* is expressed in the SHF and is important for its normal expansion[31,32]. Other genes within the 22q11 critical region probably also contribute to the syndrome. Indeed, a deficiency in one such gene, *Crkl*, results in similar defects in a mouse model and exacerbates deletion of *Tbx1* (refs 33, 34).

The known network of interacting cardiac transcription factors has continued to grow in size and complexity with the identification of the Spalt-family gene *SALL4* as the causative gene in Okihiro syndrome — which includes congenital heart diseases and limb defects almost identical to those in HOS[35,36] — and the identification of *TBX20* mutations in families with ASDs, VSDs, valve defects and impaired chamber growth[37]. SALL4 interacts physically and genetically with TBX5 to pattern the interventricular septum in a mouse model[38]. Whereas TBX5 and SALL4 can function together either to repress or to activate gene expression (depending on the target gene), TBX5, GATA4 and NKX2-5 function together

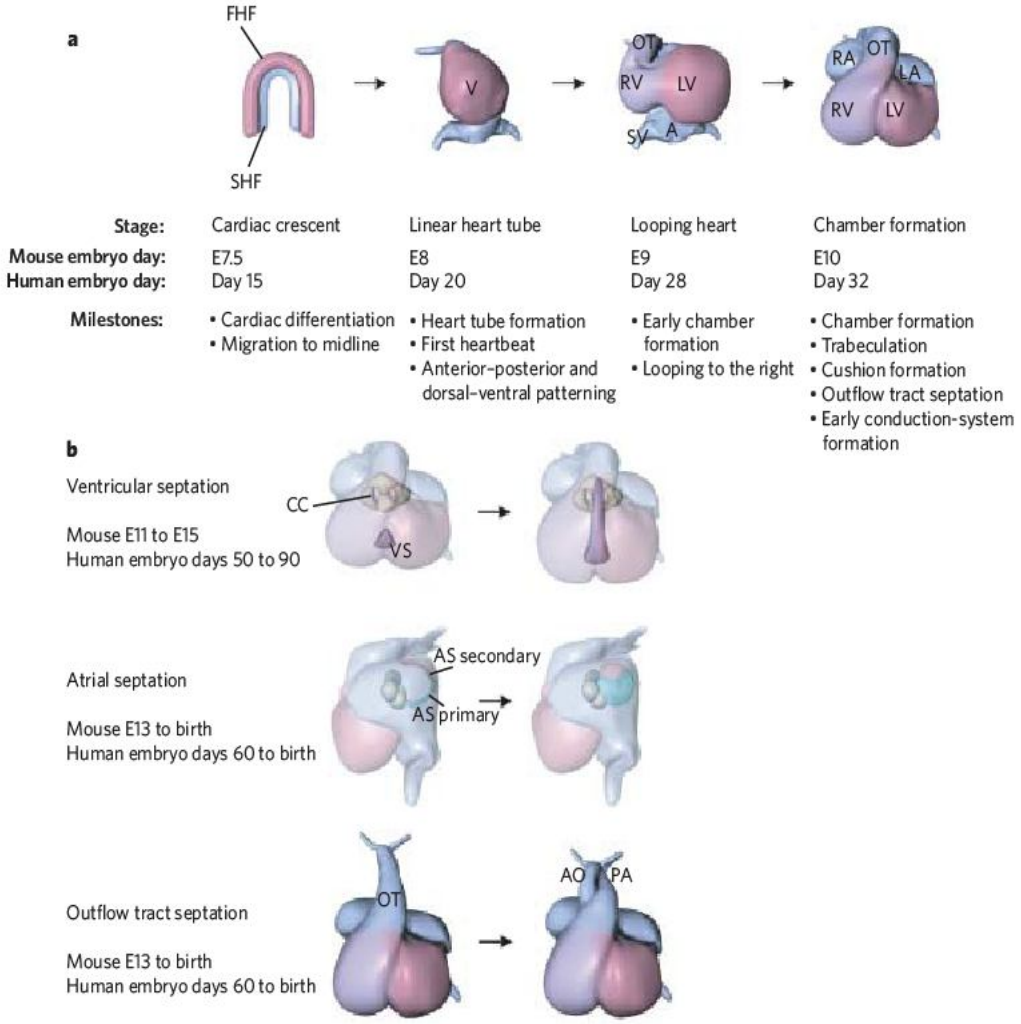

Figure 2 | Heart development. **a**, Early steps in heart development. Diagrams of heart development are shown in ventral views. At the earliest stages of heart formation (cardiac crescent), two pools of cardiac precursors exist. The first heart field (FHF) contributes to the left ventricle (LV), and the second heart field (SHF) contributes to the right ventricle (RV) and later to the outflow tract (OT), sinus venosus (SV), and left and right atria (LA and RA, respectively). V, ventricle. **b**, Maturation of the heart. The cardiac cushions (CC) will give rise to the atrioventricular valves. The ventricular septum (VS) arises from myocardium from the left and right ventricles. Atrial septation (AS) occurs by the growth of two septa: the primary septum (green) and the secondary septum (pink). Outflow tract septation separates the common outflow tract (OT) into the aorta (AO, connected to the left ventricle) and the pulmonary artery (PA, connected to the right ventricle). (An interactive version of the figure can be found at http://pie.med.utoronto.ca/HTBG/index.htm.) (Images courtesy of F. Yeung, University of Toronto, Canada.)

only to activate genes. The overlapping expression patterns and complex interactions of these transcription factors allow fine regulation of cardiac gene expression and morphogenesis[38].

Mutations in *TFAP2B*, which encodes the transcription factor activating enhancer-binding protein-2β (AP2β) and is expressed by neural crest cells, have been linked to PDA in families with Char syndrome, implying that regulation of neural-crest function is important for normal ductus closure[39]. However, the function of AP2β in heart development is unknown. Also, mutations in the gene encoding thyroid-hormone-receptor-associated protein 2 (THRAP2) — a subunit of the mediator complex, which is essential for transcriptional activation — have been reported in both a family with TGA and in sporadic cases of TGA[40], but little is known about this gene or how it functions in outflow tract development.

Although the concept that transcription factors participate in a complex set of interactions has been important for understanding the regulation of cardiac gene expression, as well as the aetiology of congenital heart diseases and their patterns of inheritance, few downstream targets have been identified that might explain the precise cellular basis for congenital heart diseases. The main challenge now is to identify the specific targets and cellular mechanisms that are involved in congenital heart diseases downstream of the associated transcription factors.

Altered haemodynamics

Complex congenital heart diseases with an outflow tract defect, such as TOF, can be accompanied by 'accessory' congenital heart diseases, such as persistent right-sided aortic arch, which can markedly alter heart physiology. Because the heart functions during its morphogenesis, haemodynamic forces might participate in cardiac morphogenesis, providing an explanation for how a primary outflow tract defect can lead to secondary structural defects. In zebrafish, altering haemodynamics mechanically or genetically has profound consequences on heart morphology[41,42]. In mice, a recent study pinpointed altered haemodynamics as a key intermediate between altered outflow tract morphogenesis and signalling events in branchial-arch artery remodelling[43]. Effects on haemodynamics might also explain some puzzling genetic data regarding the presence of mutations in the gene *MYH6*, which encodes α-myosin heavy chain, in families with inherited ASD[44]. It is unclear how defects in a gene encoding a contractile protein cause ASDs, but altered haemodynamics during embryonic development is probably a crucial factor.

Signalling defects underlie valve disease

Several types of congenital heart disease involve valve defects of varying severity. Valve dysfunction might not be severe in the infant but can often progress during adulthood, requiring valve-replacement surgery in the adult. Cardiac valve formation relies on a complex interplay of signalling between the myocardium and the overlying endocardium, which undergoes an epithelial-to-mesenchymal transition[45]. Secreted proteins are important in this process[45], and mutations affecting signalling proteins and downstream pathways can lead to valve disease.

A notable example is the Notch signalling pathway. *NOTCH1* is expressed in the endocardium of the great vessels of the heart, where it is thought to be important for epithelial-to-mesenchymal transition and valve formation[46], and it is the causative gene in some cases of BAV[47]. Individuals with BAV can also have HLHS, aortic stenosis or other serious valvular anomalies; in many cases, these patients later develop aortic-valve calcification, a major indicator for valve replacement. Individuals with *NOTCH1* mutations have a similar spectrum of defects, including aortic stenosis, VSD, TOF and, in one patient, mitral atresia, DORV and hypoplastic left ventricle[47]. NOTCH1 also represses a bone-related pathway[47], which might explain calcifications in the cardiac valves of patients with *NOTCH1* mutations.

Alagille syndrome, which affects cardiac valves, can also result from defective Notch-pathway signalling. The causative gene in most patients

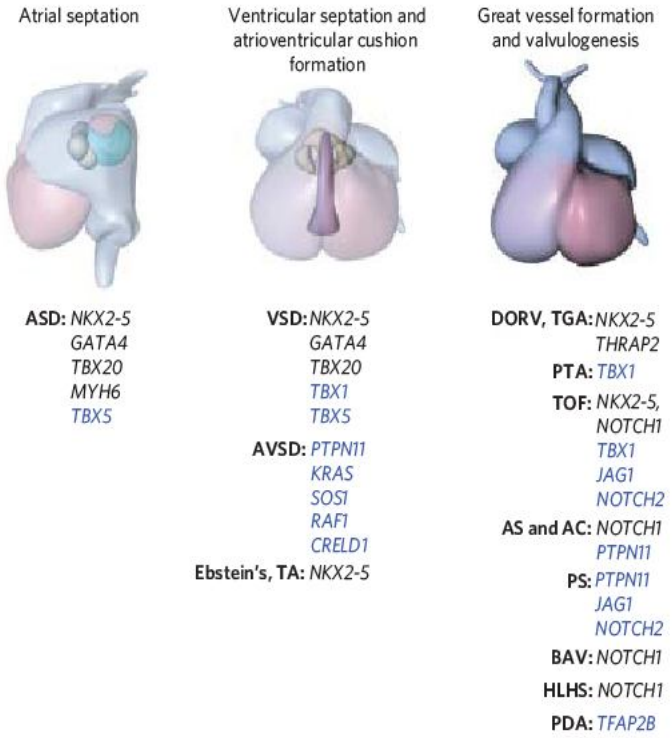

Figure 3 | Origin and genetic aetiology of congenital heart disease. Three major classes of developmental defects are indicated: defects in atrial septation, in ventricular or atrioventricular septation, and in the great vessels. The types of congenital heart disease that occur within each class are indicated, with the associated mutated genes listed. Genes for which mutations result in discrete congenital heart diseases are indicated in black; genes that are mutated in congenital heart diseases that are part of a wider syndrome (also involving defects that are not associated with congenital heart disease) are indicated in blue. *CRELD1*, cysteine-rich with epidermal-growth-factor-like domains 1; *KRAS*, ki-Ras; *PTPN11*, protein tyrosine phosphatase, non-receptor type 11; *SOS1*, son of sevenless homologue 1. (Images courtesy of F. Yeung, University of Toronto, Canada.)

with this syndrome, which includes pulmonary valvular stenosis and occasionally TOF, encodes the Notch ligand JAG1 (refs 48, 49). More recently, mutations in the gene *NOTCH2* have also been identified in families with Alagille syndrome[50], reinforcing the link between this disease and defective Notch-pathway signalling and demonstrating genetic heterogeneity of the disease.

Signalling defects also underlie valve disease in Noonan syndrome, an inherited multifaceted disease that includes various heart defects, predominantly defective pulmonary valves and AVSDs but also hypertrophic cardiomyopathy. Mutations in a set of genes that encode proteins of the Ras–mitogen-activated protein-kinase signalling pathway (SHP2, ki-Ras, RAF1 and SOS1), which regulates multiple aspects of cellular function, cause Noonan syndrome and the related cardio-facio-cutaneous syndrome[51–55]. Most of the mutations that lead to these two syndromes are activating mutations, and study of a mouse model of Noonan syndrome led to the conclusion that overactive SHP2 signalling results in hyperproliferation of outflow tract cushions[56].

MicroRNA dysfunction

In the past few years, much excitement has resulted from a newly identified class of small non-coding RNAs called microRNAs (miRNAs). These small (21-nucleotide) RNAs modulate protein function by binding to target messenger RNA, resulting in repression of translation or in degradation of the target mRNA[57]. A number of miRNAs have recently been shown to function in the heart[58]. Potentially of most relevance to congenital heart disease, miR-1 was shown to be important in the embryonic development of the heart[59,60].

Two separate genes, *miR-1-1* and *miR-1-2*, encode miR-1. Both genes are expressed in the developing heart, and transgenic overexpression experiments have suggested that these genes might be involved in regulating cardiomyocyte proliferation[59]. Both genes are under the control of serum response factor, indicating that they are part of a developmental programme regulated by cardiac transcription factors[59]. It has been shown that miR-1 targets the cardiac transcription factor HAND2, which is implicated in the growth of the embryonic heart, as well as several other regulators of cardiac growth and development. A gene-targeting approach found that deletion of *miR-1-2* results in heart defects that include VSDs; surviving mice have conduction-system defects and increased cardiomyocyte proliferation[60]. Thus, dysregulation of miR-1 or other developmentally important miRNAs might result in congenital heart disease in humans.

The later consequences of embryonic defects

Individuals with congenital heart disease can suffer from secondary heart disease later in life, possibly as a result of corrective surgery during infancy. The sequelae are sometimes severe; for example, after closure of a septal defect, some patients can progress to heart failure[61]. With improved surgical outcomes for those with congenital heart disease, the number of adults with such diseases now exceeds the number of children. Thus, it has become imperative to understand the postnatal consequences of congenital heart diseases. Recent results suggest that these might be caused, at least in part, by the direct effects of mutations associated with congenital heart disease on postnatal heart morphology and function. For example, in a family with *GATA4* mutations, apart from having heart structural defects, some individuals developed dilated cardiomyopathy later in life[27]. Indeed, data from mouse models support a connection between *GATA4* mutations and adult cardiomyopathy[62]. Similarly, mutations in *TBX20* were identified in patients with cardiomyopathy[37], as well as in those with structural congenital heart diseases.

Mouse studies have also revealed roles for other congenital-heart-disease-associated genes in cardiac function. Studies of mice in which *Nkx2-5* had been deleted only in the ventricles suggest a role for this gene in the function of the postnatal conduction system and in myocardial structure, and examination of patients with *NKX2-5* mutations revealed that some had aspects of cardiomyopathy, as predicted from the mouse data[63]. A primary defect in cardiac relaxation has been identified in a mouse model of HOS. This defect results from impaired calcium cycling owing to reduced expression of the gene *Serca2*, which encodes a calcium-uptake pump. Patients with HOS also have diastolic dysfunction (Y. H. Zhou, A. O. Gramolini, M. A. Walsh, Y. Q. Zhou, C. Slorach, M. Friedberg, J. K. Takeuchi, H. Sun, R. M. Henkelman, P. H. Backx, A. N. Redington, D. H. MacLennan and B.G.B., unpublished observations). Thus, embryonic-patterning genes control structural components of the heart and can also have a separate role in heart function, for example by regulating *Serca2*. These genes can thus modulate important aspects of heart function that cause pathology in the postnatal heart when dysregulated. This concept has important implications for the clinical management of adults with congenital heart disease.

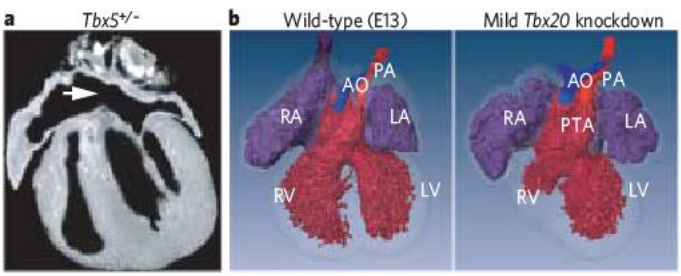

Figure 4 | Modelling human congenital heart diseases in mice, and dosage-dependent regulation of cardiac morphogenesis. a, Heart magnetic resonance imaging section of a mouse that is heterozygous for a *Tbx5* mutation, demonstrating an ASD (arrow), as also seen in humans heterozygous for a *TBX5* mutation. (Panel adapted, with permission, from ref. 25.) **b**, A partial (about 60%) reduction in TBX20 levels leads to a hypoplastic right ventricle and PTA, as also seen in humans heterozygous for a *TBX20* mutation. The outline of the heart is translucent white; the fill of the atria is purple; the fill of the ventricles and outflow tract is dark red; the aorta is blue; and the pulmonary artery is light red. E, day of embryonic development. (Panel adapted from ref. 65.)

Box 1 | The relevance of mouse models

Mice are often good models for studying human disease. But do mice carrying mutations in genes that are associated with congenital heart disease closely recapitulate the human conditions? The answer is yes for some such genes; for example, mice in which one allele of *Tbx5* has been deleted have heart defects that accurately mimic those found in patients with HOS who have a heterozygous *TBX5* mutation[25] (Fig. 4a). However, for other gene mutations that are associated with congenital heart disease, there are differences in the precise heart defects seen and in the sensitivity of the phenotype to gene dosage. For example, deletion of one allele of *Nkx2-5* in mice replicates only subtle aspects of human disease resulting from *NKX2-5* deficiency[24]. As another example, *Tbx20* deficiency in mice results in heart defects only if the dosage falls below 50% (refs 65, 66) (Fig. 4b), whereas in humans, *TBX20* mutations that are predicted to cause a 50% loss of function result in a range of structural defects[37]. Similar dosage effects on the severity of congenital heart disease can be found in mice with mutations affecting several signalling molecules, such as bone morphogenetic proteins. For example, an allelic series of *Bmp4*, in which different mutations result in varying amounts of protein being produced, shows the full spectrum of human atrioventricular canal defects, from primum ASD to complete AVSD[67], indicating that mutations affecting this pathway could cause human congenital heart disease (although this has not yet been verified). Differing sensitivities to gene dosage in mice and humans are probably an important factor in their differing manifestations of congenital heart disease. The basis of these differences in dosage sensitivity might be species-specific physiology — such as the shorter gestation time or faster heart rate of mice — or it might be influenced by genetic factors, as suggested by studies that demonstrate an effect of genetic background on congenital heart diseases[68]. Another difference between human and mouse models of congenital heart disease is that humans with defects such as PTA or DORV survive to term, whereas mice with these defects rarely do. Thus, mouse models are of considerable value for research on congenital heart diseases, but intrinsic differences between mouse and human physiology need to be carefully taken into account.

Future perspectives

The study of congenital heart diseases has come a long way since their description and classification. Improvements in *in utero* diagnosis and surgical techniques have considerably brightened the prospects for infants born with congenital heart diseases, but biological insights into this set of developmental diseases have been gained only recently. Identification of the causative genes in inherited forms of congenital heart disease has pointed towards specific pathways of disease and, in the process, provided considerable new knowledge about heart development.

Many issues remain to be addressed, however. Although transcriptional programmes that are impaired in individuals with congenital heart diseases are being identified, the mechanisms of how these deficiencies translate to a structural defect are unknown. For example, what cellular events are defective in the pathogenesis of TGA? A recent clue comes from the finding that WNT11, which signals downstream of the transcription factor PITX2, regulates morphogenesis of the outflow tract through transforming growth factor-β2, resulting in altered cell shape[64]. Another question is how epigenetic regulation coordinates these genetic programmes into a cohesive whole, and why a mechanism so sensitive to dosage perturbation has been maintained throughout evolution. Now that some of the genes involved in the main forms of congenital heart disease have been identified, a new challenge is to understand how common polymorphisms in these genes might cause subtle, yet more prevalent, disease. Finally, with the success of surgical interventions for many congenital heart diseases, what are the consequences of such diseases for adults, and how should these patients be evaluated and treated? With the current progress in understanding congenital heart diseases and heart development, the next ten years are likely to provide much clearer answers to these questions. ■

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The search for new cardiovascular biomarkers

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Despite considerable advances in the treatment of cardiovascular disease, it remains the leading cause of death in developed countries. Assessment of classic cardiovascular risk factors — including high blood pressure, diabetes and smoking — has a central role in disease prevention. However, many individuals with coronary heart disease (a narrowing of the blood vessels that supply the heart) have only one, or none, of the classic risk factors. Thus, new biomarkers are needed to augment the information obtained from traditional indicators and to illuminate disease mechanisms.

From a clinical perspective, biomarkers have a variety of functions, which correspond to different stages in the development of a disease. Biomarkers can assist in the care of patients who have no apparent disease (screening biomarkers), those who are suspected to have disease (diagnostic biomarkers) and those with overt disease (prognostic biomarkers). At present, diagnostic and prognostic cardiovascular biomarkers are available, but there are no widely accepted biomarkers for screening. This has been an active area of investigation, because preventing events in those at risk of cardiovascular disease is likely to have a substantial impact on the overall public-health burden. In this progress article, we discuss the search for clinically useful biomarkers, focusing on how emerging technologies are being integrated into current efforts.

Current status of cardiovascular biomarkers

Circulating biomarkers that have been successfully incorporated into cardiology practice fall into the category of diagnostic biomarkers: troponin I and troponin T for myocardial infarction (heart attack) and brain natriuretic peptide (BNP) for heart failure. These biomarkers also seem to have prognostic value in patients presenting with acute myocardial infarction or heart failure, and studies are ongoing to assess whether the biomarkers could be used to guide specific treatment decisions. Prognostic biomarkers might also have valuable roles as surrogate end points for therapy or clinical trials.

Several potential screening biomarkers have attracted attention because of their ability to predict future cardiovascular events and their mechanistic involvement in atherosclerosis-associated pathways. These include biomarkers associated with inflammation (C-reactive protein, interleukin-6 and lipoprotein-associated phospholipase A_2), haemostasis/thrombosis (fibrinogen and plasminogen-activator inhibitor 1), neurohormone activation (renin and BNP), insulin resistance (insulin and haemoglobin A1C) and endothelial dysfunction (homocysteine and urinary albumin). The value and appropriate use of these biomarkers remain a source of debate, however[1].

A recent investigation from the Framingham Heart Study illustrates some of the uncertainties surrounding the use of the available screening biomarkers in ambulatory individuals. The study evaluated 10 cardiovascular biomarkers in more than 3,000 people who were followed for nearly a decade[2]. Several biomarkers were found to be significant predictors of death (C-reactive protein, BNP, urinary albumin, renin and homocysteine) or cardiovascular events (BNP and urinary albumin). When biomarkers were combined into a 'multimarker' score, individuals with high scores had a fourfold higher risk of death and a twofold higher risk of major cardiovascular events than people with low scores. However, the multimarker score was associated with only a moderate increase in the area under the receiver-operating-characteristic curve (AUC) compared with a risk score based on conventional risk factors alone (Fig. 1). The AUC incorporates two features of a screening test — sensitivity and specificity — and is an objective measure of the test's ability to distinguish between individuals with and without disease. Although complementary metrics for evaluating new risk markers exist, including model calibration and reclassification percentage, these findings suggest that current screening biomarkers add only moderately to the ability of classic risk factors to predict future events in an individual person.

Limitations of the available screening biomarkers warrant consideration. As is the case for any biological analyte, biomarker concentrations are broadly distributed. Thus, even if the underlying distribution differs according to disease status, concentrations in individuals with and without disease overlap substantially[3]. Moreover, most current biomarkers participate in pathways that are known to be associated with atherosclerotic cardiovascular disease, such as those involved in inflammation and cholesterol biosynthesis. Consequently, the available biomarkers provide information that is often correlated with what is already known or being measured. Although correlated biomarkers can underscore the importance of a biological pathway, they might not provide a substantial increase in predictive value. This point is illustrated by Margaret Pepe and Mary Lou Thompson[4], who carried out simulations using two hypothetical cancer biomarkers. Assuming an AUC of 0.80 with one biomarker, they showed that the inclusion of a second biomarker raised the AUC to 0.88 if the two biomarkers were weakly correlated but only to 0.83 if the two biomarkers were moderately correlated. This result translates into a sensitivity of 80% with two weakly correlated biomarkers but a sensitivity of 70% with two moderately correlated biomarkers (assuming a false positive rate of 20%). The implication is that an additional 10 individuals for every 100 people destined to develop disease would be identified with the use of less-correlated biomarkers, a clinically meaningful difference. The difference would be further magnified if multiple biomarkers were included, emphasizing that a large number of correlated biomarkers is substantially less informative than a small number of uncorrelated biomarkers.

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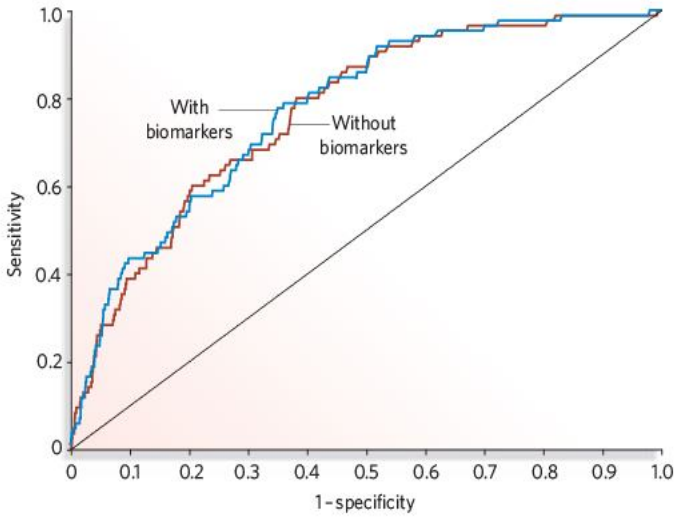

Figure 1 | Receiver-operating-characteristic curves for the prediction of cardiovascular events. The curves depict results using risk prediction models that include classic cardiovascular risk factors alone or with multiple biomarkers (that is, incorporating the multimarker score). The diagonal line denotes an uninformative test, with an AUC of 0.50. A test with perfect discrimination yields an area under the receiver-operating-characteristic curve of 1.0. Sensitivity refers to the proportion of diseased individuals with a positive test (the true positive rate). Specificity refers to the proportion of non-diseased individuals with a negative test (the true negative rate). (Reproduced, with permission, from ref. 3).

Despite the desirability of using multiple biomarkers, there are barriers to identifying new biomarkers, particularly for screening or prognostic uses. One difficulty is the requirement for large, adequately powered clinical studies. Large studies are necessary because the predictive effects of new biomarkers might be smaller than those observed with classic risk factors and because multiple biomarkers are often studied concurrently. Meta-analyses of individual participant data from multiple cohorts provide a potentially valuable tool for circumventing sample-size limitations in single cohorts.

Genetics and transcriptomics

The limitations of the available biomarkers for screening or prognostic uses underscore the importance of identifying 'orthogonal' (that is, uncorrelated) biomarkers associated with new disease pathways. Most available biomarkers have been developed as an extension of targeted physiological studies, investigating known pathways such as those involved in inflammation or haemostasis. By contrast, emerging technologies are beginning to allow the systematic, unbiased characterization of variation in genes, RNA, proteins and metabolites associated with disease conditions (Fig. 2).

Genetic studies will undoubtedly identify variants that could be biomarkers themselves or will point to circulating markers for further exploration. Current technology allows the examination of hundreds of thousands of single-nucleotide polymorphisms (SNPs) in affected and unaffected individuals to search for significant associations with disease. Although numerous studies have reported associations between variants in candidate genes and coronary heart disease, these findings have almost uniformly failed to be reproducible in subsequent analyses[5]. In contrast to candidate gene studies, genome-wide association studies provide an unbiased scan of genomic sequence variants, an approach likely to reveal new disease-associated pathways. Using this method, three groups recently identified a locus on chromosome 9p21 that is associated with early-onset myocardial infarction[6–8]. The chromosomal region identified did not contain genes recognizably associated with established risk factors for coronary heart disease such as plasma lipoproteins, hypertension or diabetes. Intriguingly, the genes encoding the cyclin-dependent kinase inhibitors INK4A and INK4B — which are known to affect cellular senescence, apoptosis and stem-cell function — are located near this chromosomal region. A recent genome-wide association study with more direct implications for the development of plasma biomarkers identified variants in the gene encoding the chemoattractant cytokine CXCL12 in individuals with premature atherosclerosis[6]. CXCL12 is involved in a variety of pathways, including cardiac development, platelet activation and stem-cell recruitment[9–11]. In the absence of expression and functional data about CXCL12 variants, however, speculation on the biological importance of this finding remains at its earliest stages.

Genetic data also provide an opportunity to assess the causality of biomarkers for disease. For a biomarker that has a causal role, the expected random distribution in a population of a polymorphism that determines high or low biomarker concentrations would be skewed in individuals, depending on their disease status. Data from 'mendelian randomization' studies are accumulating for several biomarkers such as C-reactive protein, fibrinogen and homocysteine[12,13].

The relative maturity of transcript-profiling techniques, which have been used successfully for cancer diagnostics, has led to their integration into the cardiovascular biomarker field. The application of transcriptional approaches towards the identification of new cardiac biomarkers in humans is, clearly, limited by the availability of the most relevant tissue, the heart. This difficulty has been circumvented in several recent studies[14,15]. For example, Richard Lee and colleagues[14] discovered that *ST2* messenger RNA is markedly upregulated in cultured cardiomyocytes after applying mechanical strain, an *in vitro* model that recapitulates some aspects of human pathophysiology. ST2 is a member of the interleukin-1-receptor family and exists in two forms: a membrane-bound receptor, and a truncated receptor that is soluble and can be detected in human serum. The *in vitro* data of Lee and colleagues[14] suggest that ST2 might be produced in conditions of myocardial overload such as congestive heart failure. Indeed, soluble ST2 serum concentrations predict outcomes in patients with heart failure, and an increase in soluble ST2 concentrations over time is associated with worsening prognosis[16]. Furthermore, concentrations of soluble ST2 also predict mortality and heart failure in patients after myocardial infarction[17].

In addition to the limitation of obtaining relevant tissue from myocardium or blood vessels, another obstacle to discovering biomarkers of acute heart disease is that the biomarkers identified might reflect pathological mechanisms that are associated not with events that trigger acute disease (for example, plaque rupture and thrombosis in the case of myocardial infarction) but, instead, with the downstream consequences of the resultant pathology. To address this potential pitfall, Daniel Simon and colleagues[15] profiled genes expressed by circulating platelets, which lack nuclear DNA but retain megakaryocyte-derived mRNAs and the translational machinery for protein biosynthesis. Transcriptional profiling of platelets can thus provide a window on gene expression that preceded the onset of acute events such as myocardial infarction.

Using this approach, one of the strongest discriminators between patients with acute myocardial infarction and those with stable coronary heart disease was the secreted protein myeloid-related protein 14 (MRP14). The diagnostic utility of MRP14 was validated in a prospective, nested, case–control study among apparently healthy women to assess the association of plasma MRP14 with the risk of future cardiovascular events, including myocardial infarction, stroke and cardiovascular-associated death[15]. In this study, the risk of a first cardiovascular event increased with each quartile of MRP14 concentration such that women with the highest concentrations had a fourfold higher risk of any cardiovascular event. The risk conferred by increasing MRP14 concentrations was independent of classic risk factors and C-reactive protein concentration. Thus, study of the platelet transcriptome led to the identification of a biomarker that can predict the risk of future cardiovascular events in healthy individuals.

Proteomics and metabolomics

Of the emerging platforms for biomarker discovery, perhaps none has garnered more recent attention than proteomics and metabolomics.

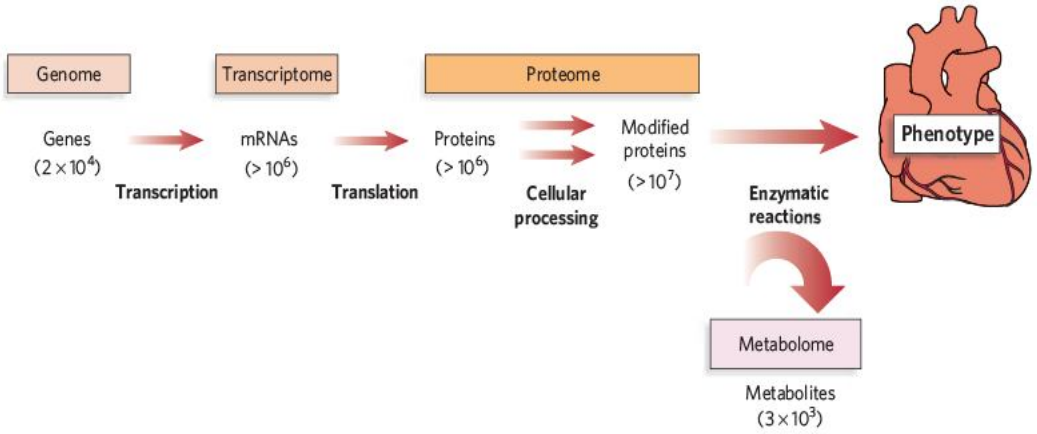

Figure 2 | The conceptual relationship of the genome, transcriptome, proteome and metabolome. Informational complexity increases from genome to transcriptome to proteome. The estimated number of entities of each type of molecule in a typical cell is indicated in parentheses.

Although still in their infancy compared with other approaches, these technologies offer complementary insight into the full complexity of the disease phenotype (Fig. 2). The set of proteins and metabolites in a cell can change rapidly in response to environmental cues, so the proteome and metabolome — the latter being defined as biochemicals, including lipids, sugars, nucleotides, amino acids and related amines, of less than 2 kDa — reflect the state of a cell or group of cells at a given time.

This complexity presents an analytical challenge, particularly as it applies to searching for biomarkers in the blood. Many cell types contribute to the plasma proteome and metabolome, which have so far been poorly characterized. In the case of the plasma proteome, the 22 most abundant proteins, including albumin and the immunoglobulins, constitute 99% of the total proteome mass[18]. Many of the biologically interesting molecules relevant to human disease are low-abundance proteins. For example, cardiac biomarkers such as troponin are found in the nanomolar range, insulin in the picomolar range, and tumour-necrosis factor in the femtomolar range. There are estimated to be tens of thousands of unique protein species in the plasma, with concentrations spanning a range of more than ten orders of magnitude. Indeed, it has been suggested that the entire set of polypeptides resulting from alternative splicing and post-translational modifications is represented in the plasma proteome in humans (estimated at more than 300,000 species)[18]. This is because the protein content of plasma includes proteins of all functional classes and from apparently all cellular localizations — most of the low-abundance proteins in plasma are intracellular or membrane-associated proteins that are present in plasma as a result of cellular turnover[19]. Recent estimates suggest that the human metabolome comprises about 3,000 small molecules and is thus more tractable than the human proteome, although in the absence of definitive data sets this remains speculative. Recently, collaborative efforts have been organized to catalogue the plasma metabolome[20].

Two core technologies have emerged as the workhorses of plasma metabolite profiling: nuclear magnetic resonance (NMR) spectroscopy and tandem mass spectrometry. NMR spectroscopy, which is used almost exclusively for analysing small biochemicals in the blood, requires relatively little sample preparation and is non-destructive, allowing further analyses. However, the method tends to have low sensitivity and can detect only highly abundant analytes. By contrast, tandem mass spectrometry, coupled with liquid chromatography, has a much higher sensitivity for both small molecules and peptides and is also applicable to a wide range of biological fluids (including serum, plasma and urine). Recent advances in tandem mass-spectrometry technology are now enabling researchers to determine analyte masses with such high precision and accuracy that peptides and metabolites can be identified unambiguously even in complex fluids.

These technologies can be used to characterize biological fluids in either a targeted manner or a pattern-discovery (fingerprint) manner. In the former, the investigator targets a predefined set of analytes to be quantified. In the latter, the investigator is faced with a complex pattern of peaks, and the molecular identities of the species giving rise to many of these peaks are generally not known. The targeted approach is more limiting than the pattern-discovery approach; however, the analysis of the data is more straightforward in this case, because the analytes giving rise to the signals are already known. By contrast, the pattern-discovery approach is inherently less biased; however, the unambiguous identification of the peaks can be laborious and difficult, and the observed associations might be spurious.

Future directions

The application of mass spectrometry and related techniques to biomarker discovery is based on decades of intensive efforts to understand and diagnose congenital errors of metabolism in infants. David Millington and colleagues[21] pioneered the use of methods based on tandem mass spectrometry for monitoring fatty-acid oxidation products, as well as organic acids and amino acids. Their work has culminated in universal neonatal screening for metabolic disorders in many geographic locations[22], allowing the identification of infants with fatty-acid oxidation disorders, organic acidaemias and aminoacidopathies. In many instances, rapid identification of these disorders triggers intervention in the form of dietary modulation, with beneficial therapeutic effects. A global metabolomic or proteomic analysis in more common diseases might similarly spotlight pathways that could be modulated by diet or drugs.

The application of proteomics or metabolomics to common cardiovascular diseases has potential obstacles, however. For acute events, such as myocardial infarction, the inherent unpredictability of when the event occurs often precludes prior blood sampling. Furthermore, the effects on biomarker concentrations are likely to be far more subtle than in the case of congenital errors of metabolism, and the extent of interindividual variability of the human proteome and metabolome remains unclear. It is clear, however, that this variability can be further compounded by environmental factors, including drug exposures. Indeed, one report applied pattern-discovery techniques to proton NMR spectra of human serum to aid in the non-invasive diagnosis of chronic coronary heart disease[23]. However, the pattern of metabolites that generated several of the spectroscopy peaks ultimately proved to be confounded by statin therapy[24,25]. Although studying samples from large patient cohorts, stratified by known risk factors or exposures, could minimize the impact of confounding clinical variables, the throughput of the detection technologies is not yet adequate for such analysis. An initial strategy to overcome such problems is to focus on well-characterized individuals who are given a physiological challenge — for example physical exercise[26] or glucose loading — and sampled over time. These individuals can thus function as their own biological control, and this type of dynamic analysis is likely to prove more reliable than analyses based on static phenotypes. Biomarkers derived from smaller, carefully phenotyped cohorts can subsequently be validated in large, more heterogeneous populations.

The identification of new biomarkers of cardiovascular disease will depend on the complementary power of genetics, transcriptional profiling, proteomics and metabolomics. The ultimate test for new

biomarkers will be to ask whether, when combined with existing clinical risk factors, they improve the prediction of risk in an individual and hence can contribute to personalized medicine. In addition to screening biomarkers, diagnostic biomarkers are needed to aid the difficult diagnoses of acute events such as reversible myocardial ischaemia, pulmonary embolism and aortic dissection. It is a long journey towards the identification of a clinical biomarker and an arduous transition from the research environment to routine clinical practice. However, there is a clear mandate for harnessing emerging technologies to systematically assess variation in genes, RNA, proteins and metabolites, as well as for identifying orthogonal biomarkers, which are unlikely to be found by focusing on well-studied pathways. ■

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Imaging of atherosclerotic cardiovascular disease

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Atherosclerosis is characterized by thickening of the walls of the arteries, a process that occurs slowly and 'silently' over decades. This prolonged course of disease provides a window of opportunity for diagnosis before symptoms occur. But, until recently, only advanced atherosclerotic disease could be observed. Now, developments in imaging technology offer many enticing prospects, including detecting atherosclerosis early, grouping individuals by the probability that they will develop symptoms of atherosclerosis, assessing the results of treatment and improving the current understanding of the biology of atherosclerosis.

Despite considerable therapeutic advances over the past 50 years, cardiovascular disease is the leading cause of death worldwide. This is mainly a result of the increasing prevalence of atherosclerosis, owing to the ageing population, the improved survival of patients with atherosclerotic cardiovascular disease and, above all, the widespread under-recognition and undertreatment of individuals with risk factors for atherosclerosis. Atherosclerosis is characterized by the thickening of the arterial wall to form an atherosclerotic plaque, a process in which cholesterol deposition, inflammation, extracellular-matrix formation and thrombosis have important roles[1] (Fig. 1) (see pages 904 and 914). Symptoms occur late in the course of disease and are usually caused by the narrowing of the lumen of the artery, which can happen gradually (as a result of progressive plaque growth) or suddenly (as a result of plaque rupture and, subsequently, thrombosis). The resultant decrease in blood supply can affect almost any organ, although coronary heart disease and stroke are the most common consequences.

Traditionally, diagnosis of atherosclerosis was possible only at advanced stages of disease, either by directly revealing the narrowing of the arterial lumen (stenosis) or by evaluating the effect of arterial stenosis on organ perfusion. However, new imaging approaches allow the assessment not only of the morphology of blood vessels but also of the composition of the vessel walls, enabling atherosclerosis-associated abnormalities in the arteries (including the coronary arteries) to be observed, down to the cellular and molecular level in some cases. Some of these approaches are now in clinical use or are being tested in clinical trials, whereas others are better suited to basic and translational research. Here, we discuss recent advances in imaging cardiovascular atherosclerotic disease, including revealing both the primary changes, in the blood vessel wall, and the secondary changes, in the structure and function of the heart. We focus first on advances in computed tomography (CT) and magnetic resonance imaging (MRI) and then discuss the growing field of molecular imaging.

The heart

Cardiac function, perfusion and contractility can be assessed non-invasively by using various techniques: ultrasound, single-photon-emission CT (SPECT), positron-emission tomography (PET) and, more recently, MRI. These imaging techniques all provide information with diagnostic and prognostic value, and their strengths and limitations have been reviewed recently[2].

MRI, in particular, has emerged as a versatile technique that can be used to assess multiple cardiac parameters non-invasively in a single session. These parameters include cardiac structure and function, metabolic status, the presence of regions lacking sufficient blood flow (ischaemic regions), and coronary artery stenosis[3]. At present, MRI is considered to be the most accurate modality for assessing the volume, mass and ejection fraction of both the left ventricle and the right ventricle, parameters with important prognostic implications. MRI can also detect changes in the magnetic properties of the tissue that are associated with increased water content; this allows imaging of myocardial oedema, which occurs in acute ischaemic injury. In addition, MRI can capture the accumulation of gadolinium ion (Gd^{3+})-based contrast agents that occurs in areas of myocardial scarring and/or necrosis within a few minutes of administration (referred to as delayed enhancement), allowing myocardial infarction to be imaged with unsurpassed resolution. The proportion of the myocardium showing delayed enhancement inversely correlates with the likelihood of dysfunctional myocardial segments recovering contractility. Recovery can occur spontaneously or through revascularization, processes that are indicative of heart injury known as 'stunning' and 'hibernation', respectively[4]. In a recent study, the detection of even small amounts of myocardium showing delayed enhancement in patients without known myocardial infarction was identified as the best predictor of future adverse cardiac events and death, in comparison with other commonly used clinical indices[5]. Moreover, because MRI provides highly reproducible results and does not involve ionizing radiation, it can be used serially in animal or human studies to test the effects of therapeutic interventions on the myocardium *in vivo*; such testing therefore requires fewer individuals than for other imaging techniques[6]. On the basis of these capabilities, MRI of the heart, either alone or in combination with other imaging modalities, could be important for assessing the potential benefits of myocardial regenerative therapy (see page 937).

The coronary arteries

The narrowing of non-cardiac arteries has traditionally been detected non-invasively by using techniques such as ultrasound, CT or MRI. CT is well suited to studying all vascular regions, although it requires the use

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of potentially nephrotoxic contrast agents and ionizing radiation. These limitations can now be largely overcome by using whole-body magnetic resonance angiography, which can be carried out in less than 90 s. This technique allows stenoses to be detected in the entire arterial system — except the coronary circulation — in a single examination[7].

Until recently, imaging of coronary stenoses required the insertion of a catheter into the coronary artery during X-ray angiography. In the past decade, however, it has become possible to image the coronary arteries non-invasively, by using contrast-enhanced CT. CT technology has evolved from machines that needed about 300 s to obtain a single image to multidetector CT (MDCT) scanners that can simultaneously acquire 256 'slices' in less than 250 ms, providing a complete coronary angiogram in less than 15 s. In selected patients with stable disease and a normal cardiac rhythm, MDCT has a sensitivity of 96% and a specificity of 74% for detecting significant coronary stenoses (defined as more than 50% narrowing of the diameter of the artery) compared with the traditional, invasive technique, catheterization, which is the gold standard[8]. From a clinical perspective, the most important advantage of MDCT is its high negative predictive value: that is, a normal result on an MDCT exam can convincingly rule out the possibility that significant coronary disease is present[9]. One limitation is that the heart rate must be slow for the image to be of adequate quality, but this might be overcome by using the newest generation of CT equipment, in which two X-ray sources and detectors are present in a single scanner (known as dual-source CT), thereby improving the temporal resolution of images[10].

The arterial walls

Atherosclerosis is a disease of the blood vessel wall, so the ability to identify plaques before luminal stenosis develops is the cornerstone of early disease detection. However, the thinness of the normal vessel wall (< 1 mm for most arteries) presents a huge challenge for imaging. Several invasive (catheter-based) techniques have been used to evaluate the morphology of plaques and other features of the vessel wall. These techniques include angioscopy (direct visualization of the inner surface of the vessel wall by using fibre-optic technology), optical coherence tomography, thermography, near-infrared spectroscopy, intravascular MRI and, most extensively, intravascular ultrasound[11]. These modalities are suitable for evaluating the coronary arteries and — because of the proximity of the imaging probe to the vessel wall — provide high spatial resolution (for example, < 15 μm with optical coherence tomography). However, the requirement for catheterization is a definite limitation. Ultrasound can also be used non-invasively to measure the intima-media thickness of the carotid arteries, because these arteries are located superficially. Increased carotid intima-media thickness provides some additive information to conventional risk factors in determining the risk of future myocardial infarction or stroke[12].

With recently developed CT technology, the coronary arteries can now be imaged non-invasively (as described earlier). CT has, however, long been used for the non-invasive detection of coronary calcium deposits (yielding a 'calcium score'), a specific indicator of atherosclerosis that has prognostic value in asymptomatic individuals. Depending on the

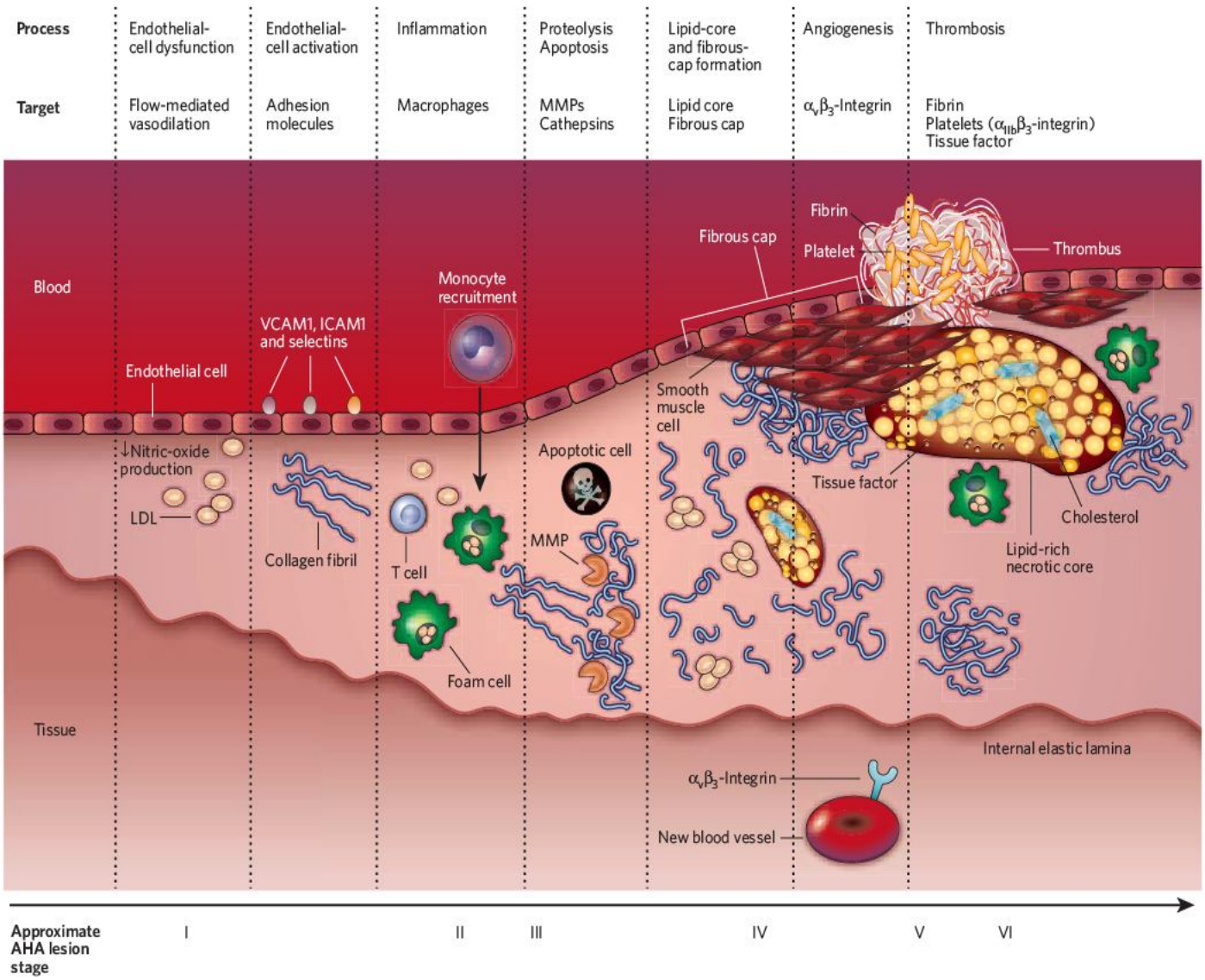

Figure 1 | The development of an atherosclerotic lesion. The progression of an atherosclerotic lesion is shown in a simplified form, developing from a normal blood vessel (far left) to a vessel with an atherosclerotic plaque and superimposed thrombus (far right). Potential targets for molecular imaging at each stage are also listed. AHA, American Heart Association; ICAM1, intercellular adhesion molecule 1; LDL, low-density lipoprotein; MMP, matrix metalloproteinase; VCAM1, vascular cell-adhesion molecule 1. Figure adapted, with permission, from ref. 25.

Table 1 | Targets and contrast agents for molecular imaging of atherosclerosis

Target	Biological roles	Contrast agent*	Rationale for use	Modality
Inflammatory cells (including macrophages)	Crucial in early plaque development, lipid modification, smooth muscle cell proliferation, extracellular-matrix formation, angiogenesis, plaque rupture and thrombosis	Ultrasmall superparamagnetic iron-oxide particles[29]	Phagocytosed by macrophages	MRI
		[^{18}F]Fluorodeoxyglucose[32,33]	Taken up by metabolically active cells	PET
		N1177 (iodinated particle)[31]	Accumulates in macrophages	CT
		CD204-specific-antibody-carrying, Gd^{3+}-loaded micelles[30]	Target the macrophage scavenger receptor (CD204)	MRI
		^{99m}Tc-labelled interleukin-2	Binds to activated T cells	SPECT
Apoptotic cells	Release pro-coagulant and pro-oxidative stimuli that contribute to plaque destabilization and myocardial injury	^{99m}Tc-labelled annexin V	High affinity for phosphatidylserine, a molecule present at the surface of apoptotic cells	SPECT
		Annexin-V-crosslinked iron oxide–Cy5.5		MRI and NIRF
Proteases	Participate in plaque remodelling and rupture, as well as in infarct remodelling and healing	Gelatinase probe	Fluorescent substrate for MMP2 and MMP9	NIRF
		Prosense[37]	Fluorescent substrate for cathepsins	NIRF
		P947	Contains an MMP-inhibitory peptide	MRI
Vascular cell-adhesion molecules	Mediate the adhesion of inflammatory cells to the endothelium and the recruitment of these cells into atherosclerotic lesions and injured myocardium	VINP-28 (ref. 35)	Contains a peptide with high affinity for VCAM1	MRI and NIRF
		Microbubbles containing sialyl-LewisX (ref. 36)	Bind to selectins	Ultrasound
Extracellular matrix	An important component of plaques, particularly abundant in advanced lesions	Gadofluorine	Binds to extracellular-matrix components (such as tenascin, proteoglycans and collagen)	MRI
Lipoproteins	Involved in the trafficking of cholesterol between the blood and atherosclerotic plaques	^{99m}Tc-labelled MDA2	Binds to oxidized LDL, a potent intraplaque pro-inflammatory stimulus	SPECT
		HDL-like nanoparticles	HDL removes cholesterol from plaques (known as reverse cholesterol transport)	MRI
New blood vessels	Contribute to intraplaque haemorrhage, plaque growth and destabilization, and myocardial healing and remodelling	Paramagnetic nanoparticles[38]	Target $\alpha_v\beta_3$-integrin, a key mediator of angiogenesis	MRI
		^{99m}Tc-labelled NC100692		SPECT
Thrombi	A hallmark of acute vascular syndromes, and promote plaque growth	EP-2104R (ref. 34)	Contains a peptide with a high affinity for fibrin	MRI
		^{99m}Tc-labelled apcitide	Contains a peptide with a high affinity for the platelet cell-surface molecule $\alpha_{IIb}\beta_3$-integrin	SPECT
		IR-786-labelled platelets[40]	Incorporated into thrombi	NIRF

*For agents for which no reference is given, and for discussion of other potential targets, see refs 22, 24–28. HDL, high-density lipoprotein; LDL, low-density lipoprotein; MDA2, monoclonal antibody specific for malondialdehyde; MMP, matrix metalloproteinase; NIRF, near-infrared fluorescence; Tc, technetium; VCAM1, vascular cell-adhesion molecule 1.

individual studied (in terms of age, ethnicity, baseline risk of cardiovascular disease, and so on) and the thresholds used, the risk of subsequent death or myocardial infarction associated with a high calcium score increases up to 12-fold after adjusting for conventional risk factors[13]. As a result, it has been proposed that the coronary calcium score, alone or in combination with the carotid intima-media thickness, could be used for initial stratification of cardiovascular risk in the general population[14]. Nonetheless, this approach is not without controversy, largely because of the required X-ray exposure and the financial cost[15]. Although coronary calcifications are easily detected by CT, about three-quarters of all coronary lesions are non-calcified plaques. Such plaques can now be detected with modern CT scanners after contrast agents have been administered to patients. Moreover, in patients with chest pain, it was recently shown that the extent of non-calcified atherosclerosis in a coronary CT angiogram is correlated with increasing mortality[16]. CT can also provide reasonably accurate quantification of plaque size and crude characterization of plaque composition, on the basis of lipid-rich tissue attenuating X-rays to a smaller extent than fibrous tissue[17].

MRI has also developed into an excellent modality for non-invasively evaluating the blood vessel wall, and it has the advantage over CT of not exposing the patient to ionizing radiation. 'Black-blood' techniques (an imaging approach in which the blood appears black and the arterial wall can be seen) accurately depict plaque presence, size and morphology with submillimetre resolution and high reproducibility, providing new indices of atherosclerotic burden that can be applied to large populations[18]. Using this technique, with serial testing of an individual, it is possible to track changes in arterial disease and to test the effects of therapies for atherosclerosis in a completely non-invasive manner[19]. Although preliminary data show that it is feasible to use MRI to evaluate the coronary artery wall, this remains challenging because of the small diameter, the tortuosity (twistedness) and the continuous movement of the coronary arteries. Therefore, MRI is mainly used to study extra-cardiac vessels. MRI can also be used to provide insight into the composition and biological activity of different types of atherosclerotic lesion, one of the most important goals of imaging. The probability of plaque rupture and the subsequent clinical complications differ substantially between plaque types. Features of higher rupture risk include the following: active inflammation, a thin fibrous cap with a large lipid core, erosion or fissure of the plaque surface, a superimposed thrombus, a stenosis that narrows the luminal diameter by more than 90%, superficial calcified nodules, intraplaque haemorrhage and outward remodelling[20]. By combining images acquired with different parameters, MRI can reliably detect and quantify plaque components such as lipids, fibro-cellular tissue, calcium and intraplaque haemorrhage, and can detect and characterize a superimposed thrombus[21,22]. The clinical implications of these capabilities were highlighted in a recent study of asymptomatic patients with moderate carotid stenosis (50–79% luminal narrowing) in which several high-risk features of the plaques observed by using MRI predicted subsequent cerebrovascular events[23].

Molecular imaging

Not only has the ability to image cardiovascular anatomy and physiology on a macroscopic scale (as has been discussed so far) improved markedly in the past decade, but it has also become increasingly possible to detect biological processes at the cellular or even molecular level. Molecular imaging relies on the use of contrast agents that target specific cells or molecular pathways of relevance to disease. In addition to the various imaging techniques being developed, contrast agents for tracking potentially important components of atherosclerotic disease

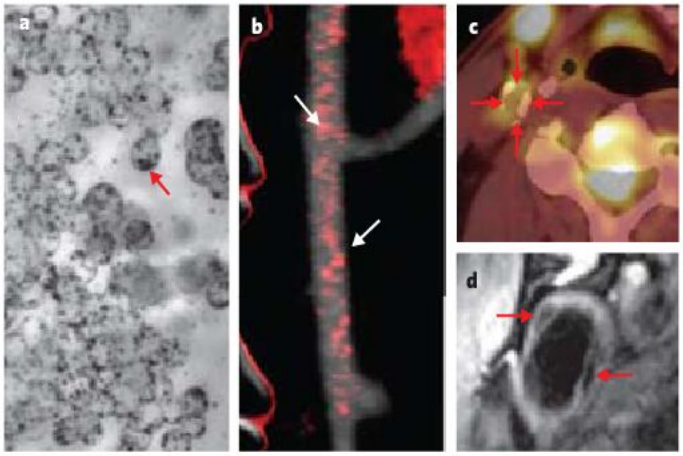

Figure 2 | Multimodal imaging of inflammation and atherosclerosis. **a**, Molecular imaging of macrophages by using CT, with the iodinated contrast agent N1177. After *in vitro* incubation of mouse macrophages with N1177, light microscopy in a phase-contrast mode shows the presence of multiple cytoplasmic granules (red arrow), confirming uptake of the contrast agent by macrophages. **b**, Three-dimensional reconstruction of a rabbit's abdominal aorta at 2 h after intravenous administration of N1177. A false-colour image of N1177 staining is shown overlaid on an angiogram. Intense red spots indicate areas of N1177 accumulation in aortic plaques, which are rich in macrophages (white arrows). Organs with high macrophage density are also visible, including the spleen (top right). **c**, A combined PET and CT image of a human neck (axial view). Atherosclerotic pathology is present at the bifurcation of the right common carotid artery (red arrows), as determined by the presence of heavy calcification and large amounts of [^{18}F]fluorodeoxyglucose, which is indicative of inflammatory activity. **d**, Black-blood MRI of the same artery. Carotid arterial wall thickening is evident, as are two areas of signal drop (red arrows), which correspond to calcified regions. (Panels **a** and **b** reproduced, with permission, from ref. 31. Panels **c** and **d** reproduced, with permission, from ref. 26.)

are at various stages of development[22,24–28] (Fig. 1 and Table 1). Most of the available probes are in experimental testing, although some have already advanced to clinical evaluation. Imaging probes typically include a moiety (such as an antibody or specific ligand) with high affinity for the desired target molecule. Alternatively, the probe can be modified to facilitate uptake by specific cells. In addition, probes are designed to be detected by various modalities, including ultrasound (which detects microbubbles), SPECT and PET (radioactive isotopes), MRI (paramagnetic and superparamagnetic compounds), CT (iodinated compounds) or optical imaging (fluorochromes). Many of the targets of interest are located in deep organs and are present at very low (nanomolar) concentrations; imaging modalities therefore need to be highly sensitive, as well as safe and economically viable.

Ultrasound is widely available, safe and inexpensive, but it has insufficient penetration for the non-invasive imaging of deep vessels (including the coronary arteries) with high spatial resolution or sensitivity. SPECT and PET have a high sensitivity, but they also have limited spatial resolution and the additional disadvantage of requiring the use of radioactive agents. By contrast, MRI has a somewhat lower sensitivity than SPECT and PET and requires prolonged imaging times, but it is safe and provides excellent resolution (~10 μm with high-field magnets). CT, conversely, offers the advantages of fast scanning times and superior performance for coronary angiography, at the expense of limited sensitivity and the use of nephrotoxic agents and ionizing radiation. Optical imaging techniques — for example, near-infrared fluorescence reflectance or fluorescence molecular tomography — have excellent sensitivity and temporal resolution and allow the tissue distribution of the probe to be precisely determined with *ex vivo* fluorescence microscopy. So far, however, such techniques can be used non-invasively only to monitor superficial structures because of the limited ability of light to penetrate tissue. Optical imaging techniques and some SPECT and MRI techniques have the advantage of being able to detect more than one molecular signature at a time.

The choice of target for imaging is also clearly important. Because inflammation has a crucial role at all stages of atherosclerosis (Fig. 1), macrophages are currently one of the most appealing targets. Ultrasmall paramagnetic iron-oxide particles are engulfed by macrophages *in vivo*, and this causes a detectable decrease in the MRI signal in proportion to the degree of atherosclerotic plaque inflammation, as shown in human studies[29]. A strong correlation between macrophage density and MRI signal was also found recently in a mouse model of atherosclerosis, by using a contrast agent consisting of Gd^{3+}-loaded micelles targeted to the macrophage scavenger receptor[30]. Similarly, in rabbits, specific uptake of an iodine-containing contrast agent by macrophages allows atherosclerotic lesions to be detected by using CT[31] (Fig. 2a, b). Also, with PET, the signal from [^{18}F]fluorodeoxyglucose correlates with the concentration of macrophages in human atherosclerotic plaques[32]. Moreover, by using specialized equipment, several imaging techniques can be used concurrently — for example, PET together with CT or, recently, MRI (Fig. 2c, d) — for the sensitive and reproducible detection of vascular inflammation[33]. This combination approach allows the most appropriate technique(s) for a particular patient, vascular region and/or disease stage to be chosen and takes advantage of the particular strengths of each modality.

Thrombi are another attractive target for imaging, because acute clinical events often occur as a result of thrombosis triggered by plaque rupture (Fig. 1). In animal models, thrombi of different ages and in different vascular regions have been detected with MRI[34], and this approach is now being investigated in humans[26]. At the other end of the timeline of atherosclerotic-plaque progression (Fig. 1), cell-adhesion molecules participate in the early development of lesions by facilitating the recruitment of leukocytes into the vessel wall. In an animal model, increased amounts of vascular cell-adhesion molecule 1 (VCAM1) were found in aortic plaques by using a dual contrast agent detectable by both MRI and optical imaging[35]. A similar approach, which used ultrasound detection of microbubbles, found increased expression of endothelial selectins in the heart of rats that had been subjected to transient myocardial ischaemia followed by reperfusion[36]. Development of such probes for clinical use could allow the identification of atherosclerosis at early stages and the detection of plaque rupture (which, even when clinically silent, indicates disease instability).

Another possibility is to use probes that emit a detectable signal only after they have been activated by the target. For example, in a recent study of an experimental model of atherosclerosis, a fluorescent probe activated by enzymatic degradation was used to reveal intraplaque protease activity with near-infrared fluorescence[37].

In addition to being diagnostic and prognostic indicators, probes could also be used for therapeutic purposes, to deliver drugs in a targeted manner. An example of this is a study in which rabbits were administered paramagnetic nanoparticles loaded with an antiangiogenic drug, resulting in a reduction in the extent of blood vessels in atherosclerotic plaques, as observed by non-invasive tracking with MRI[38]. In addition, haematopoietic or cardiac stem cells for use in cell-based therapy could be tagged with appropriate imaging probes, providing insights into the role and fate of these cells after their administration[39].

Future directions

Rapid technological progress is transforming the imaging of atherosclerotic cardiovascular disease from a method of diagnosis in symptomatic patients to a tool for the non-invasive detection of early subclinical abnormalities. In addition, a new generation of hybrid technology is now becoming available; this technology combines multiple imaging modalities in a single platform, using one machine for more than one type of imaging (Fig. 2c). And new probes designed to be detected by several modalities can take advantage of the strengths of each.

The availability of more powerful imaging techniques has the potential to improve our understanding of the biology of atherosclerosis. Much of the current knowledge has been inferred from static histopathological observations of animal models or human tissue samples studied at different disease stages or after various therapeutic interventions. By using molecular imaging, it is now becoming increasingly possible to obtain

non-invasively — from living experimental animals and, even, humans — the type of information that was previously available only through immunohistochemistry.

Improved imaging technologies also hold promise for aiding drug development. Rather than relying on the plasma concentrations of a specific therapeutic agent to infer that it has been delivered to the target organ, imaging might be able to provide a direct read-out of the agent's local concentration and activity. Such information could be enormously helpful for deciding which therapies are the best candidates for proceeding to clinical trials[26].

Prospective studies that will provide information about the significance of currently available imaging data, considered either alone or in the context of conventional risk factors and emerging serum and genetic biomarkers (see page 949), are in progress. Examples are the Multi-Ethnic Study of Atherosclerosis (MESA; http://www.mesa-nhlbi.org/) and the High-Risk Plaque (HRP) Initiative (http://www.hrpinitiative.com), both studies of asymptomatic individuals of various ethnic backgrounds. Another example is a subset of the FREEDOM trial (Future Revascularization Evaluation in Patients with Diabetes Mellitus: Optimal Management of Multivessel Disease trial), which involves diabetic individuals with proven coronary heart disease. With data from such investigations, it might be possible to distinguish which patients would benefit from therapeutic intervention. Preventing atherothrombotic events through early detection would have enormous medical impact, and imaging is set to have a prominent role in making this a reality. In this regard, it will be imperative to evaluate emerging imaging technologies rigorously to ensure that they are cost-effective. ■

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Thrombin receptor antagonist as a novel approach for the treatment of atherothrombosis

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Schering-Plough is actively engaged in the research and development of drugs for the treatment of atherothrombosis, which is the leading cause of death worldwide[1]. Characterized by atherosclerotic lesion disruption with superimposed thrombus formation, this condition is clinically manifested as sudden cardiac death, myocardial infarction, stroke, transient ischaemic attack and peripheral arterial disease. Platelets are central to the blood clot formation. Additionally, several cardiovascular risk factors have been associated with increased blood thrombogenicity. Scientists at Schering-Plough have been developing drug targets that inhibit platelet aggregation and reduce plasma low-density lipoprotein (LDL) cholesterol, including the following: the intravenous glycoprotein IIb/IIIa (GPIIb/IIIa) antagonist Integrilin (eptifibatide), developed with COR Therapeutics to reduce ischemic events in acute coronary syndromes; the cholesterol absorption inhibitor Zetia (ezetimibe) and Vytorin (a combination of ezetimibe and the 3-hydroxy-3-methyl-glutaryl-coenzyme A reductase inhibitor simvastatin), which reduce LDL cholesterol in patients with high cholesterol, marketed with co-development partner Merck for the treatment of hypercholesterolaemia; and the thrombin receptor antagonist SCH 530348, a potentially first-in-class antiplatelet agent, which is currently in phase III clinical trials for the treatment of acute coronary syndromes and secondary prevention. This article outlines the efforts of Schering-Plough in the discovery and early clinical development of thrombin receptor antagonists for the treatment of atherothrombosis.

Haemostasis and thrombosis

The ability of the body to control the flow of blood following vascular injury is paramount to continued survival. The process of blood clotting and the subsequent dissolution of the clot, following repair of the injured tissue, is termed haemostasis. This comprises a series of events that occur following the loss of vascular integrity, starting with vascular constriction that limits the blood flow to the area of injury. Next, platelets become activated and aggregate at the site of injury, forming an initially loose platelet plug. To insure stability, a fibrin mesh forms and entraps the plug. Finally, the clot must be dissolved in order for normal blood flow to resume following tissue repair. The dissolution of the clot occurs through the action of plasmin. Haemostasis is therefore sometimes referred to as 'good clotting' because it helps the body to heal following vascular injury. In physiological conditions, several inhibitory mechanisms prevent activated coagulation reactions from amplifying uncontrollably and from causing extensive local thrombosis. However, in many pathological conditions, such as those following plaque rupture, the procoagulant forces amplify the initial process leading to thrombosis — the formation of a clot inside a blood vessel, which obstructs the flow of blood through the circulatory system. Depending on the location, the thrombus can be defined as arterial or venous. Thrombi that form in arteries are predominantly platelet rich and can lead to stroke, myocardial infarction or peripheral vascular disease. Thrombi that form in veins are predominantly fibrin rich and lead to deep-vein thrombosis; they often become dislodged and transported to other vascular beds, as is the case in pulmonary embolism. Thrombosis is therefore also referred to as 'bad clotting' because it obstructs blood flow, which, when it happens within vital organs such as the heart or the brain, can lead to death.

Role of platelets in thrombosis and haemostasis

Disruption of the endothelium during vessel injury exposes the subendothelial elements, allowing adherence by otherwise quiescent circulating platelets. The initial tethering of platelets to the sites of vascular injury is mediated by the GPIb/V/IX complex; von Willebrand factor is the major ligand for this complex formation. In addition, collagen receptors with tethering function, including GPVI and GP1a, are found on the platelet surface. The initial platelet adhesion is crucial for the formation of a platelet plug. Once adhered to the collagen network, platelets undergo shape change, release the contents of their granules and, in turn, activate other platelets. This process is driven by a number of agents, including ADP, thrombin and thromboxane A_2. By acting on selective G-protein-coupled receptors (Fig. 1), these agents lead to an amplification of the initial response resulting in the formation of a haemostatic platelet plug. The GPIIb/IIIa receptor serves as the final common pathway that leads to platelet aggregation. The serine protease thrombin, which is the most potent pathophysiological activator of platelets, is generated at the sites of vascular injury and on the surface of activated platelets. Thrombin, along with ADP and thromboxane A_2 released from activated platelets, continues to drive aggregation and leads to thrombosis. Thrombin has a dual role in the context of haemostasis: in addition to activating platelets, it plays a central part in orchestrating the coagulation cascade where it cleaves fibrinogen to form fibrin.

Platelet receptors and the current target of antiplatelet drugs

Platelets have multiple activators that act on specific G-protein-coupled receptors on their surfaces and can function independently of one another (Fig. 1). Thrombin activates human platelets via novel G-protein-coupled receptors called protease-activated receptors (PARs)[2]. There are two subtypes on human platelets: PAR-1 and PAR-4. ADP activates platelets via two receptor subtypes called $P2Y_1$ and $P2Y_{12}$. Scientists at Schering-Plough were among the first to clone the $P2Y_{12}$ receptor[3]. We have also generated $P2Y_{12}$-null mice and demonstrated the crucial role of this receptor in mediating the platelet response to ADP[4]. In addition, we have shown that the $P2Y_{12}$ receptor is the target of the widely used thienopyridine drugs[4]. Thromboxane A_2 activates platelets via the thromboxane receptor. The widely used antiplatelet drug aspirin is a cyclooxygenase inhibitor that works by

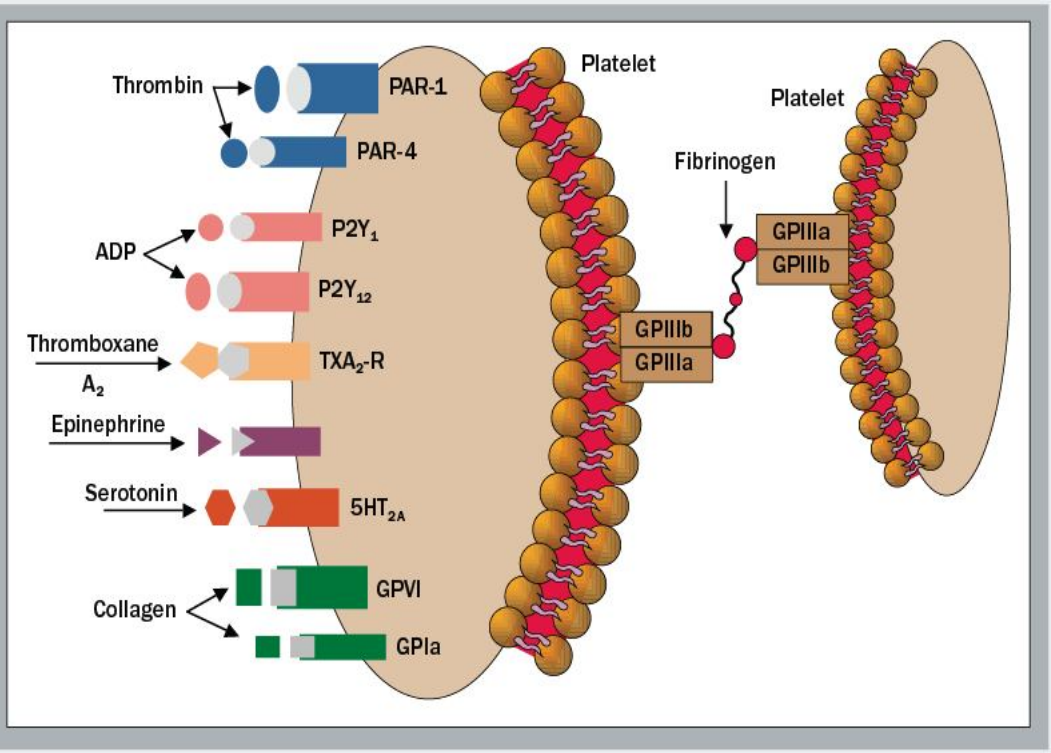

Figure 1 | Platelet receptors. Illustration of a platelet with the various activation pathways shown on the left. Upon activation by one or more of these pathways via their corresponding agonists, the GPIIb/IIIa receptor undergoes a conformational change that allows the platelet to bind to fibrinogen, thereby facilitating the bridging of activated platelets leading to aggregation and thrombus formation. $5HT_{2A}$, serotonin$_{2A}$; TXA_2-R, thromboxane A_2 receptor.

blocking arachidonic-acid metabolism and the subsequent generation of thromboxane A_2. The thienopyridine drugs, including ticlopidine and clopidogrel, irreversibly inhibit the platelet $P2Y_{12}$ ADP receptor. Eptifibatide, abciximab and tirofiban are GPIIb/IIIa inhibitors approved only for intravenous use. Eptifibatide was discovered by COR Therapeutics and co-developed by Schering-Plough. Currently, we market Integrilin in North America. The existing antiplatelet drugs available for chronic oral therapy are limited to aspirin and the ADP antagonists. Despite the aggressive use of dual antiplatelet therapy in the treatment and prevention of atherothrombosis, events continue to occur. Scientists at Schering-Plough are therefore focused on targeting the thrombin pathway on platelets as a novel approach to the treatment of arterial thrombosis. Thrombin receptors are also present on several other cell types, including endothelial cells, smooth-muscle cells, lymphocytes, neutrophils and monocytes, and thrombin is reported to play a crucial role in chemotaxis, and an inflammatory and reparative role in response to vascular injury (Fig. 2). Thrombin receptor antagonists therefore have the potential to play a broader role in preventing atherothrombosis than just inhibiting platelet activation.

Thrombin receptors and platelets

Thrombin activates human platelets via novel PARs. Thrombin binds to the extracellular loop of the receptor, and then cleaves it to create a new receptor amino terminus that functions as a tethered ligand and activates the receptor (Fig. 3). Synthetic peptides mimicking the putative new amino terminus created by thrombin cleavage are potent agonists of the thrombin receptor, and can cause platelet activation, secretion and aggregation. These findings reveal an unprecedented mechanism of receptor activation. Such synthetic peptides — also referred to as thrombin receptor agonist peptides (TRAPs) — have been useful in studying the functions of thrombin receptors. PAR-1 is the high-affinity receptor for thrombin on human platelets, whereas PAR-4 serves as the low-affinity site for thrombin[5]. Studies conducted by various groups, including scientists from Schering-Plough, have revealed species differences in the thrombin receptors on platelets. Laboratory species commonly used in studying platelet function, including rats, mice, rabbits and dogs, do not express PAR-1 on their platelets. It has been shown that rats and mice have PAR-3 and PAR-4 thrombin receptors on their platelets. However, we have demonstrated that only guinea pigs and non-human primates (including cynomolgus monkeys, rhesus monkeys and baboons) have PAR-1 receptors on their platelets[6]. We therefore determined early on that the commonly used rodent species were not viable animal models for the discovery of thrombin receptor antagonists intended for human use.

Development of a human platelet membrane-binding assay

Scientists at Schering-Plough set out to establish a binding assay using human platelet membranes. Although the commercially available TRAPs can activate PAR-1, they have low affinities for PAR-1 and were therefore not suitable for radiolabelled ligand binding assays. This necessitated us and others in the field to synthesize more potent TRAPs. Feng *et al.*[7] reported on the synthesis of a high-affinity peptide, A(pF-F)R(Cha)(hR)Y-NH_2, which exhibited an effector concentration for half-maximum response (EC_{50}) value of 10 nM for stimulation of human platelet aggregation. Using a tritiated version of this peptide, we established a high-throughput thrombin receptor-binding assay[8] with human platelet membranes to screen for leads. This jump-started our efforts to discover a selective PAR-1 thrombin receptor antagonist. Using this assay, we were able to identify a series of potent and selective compounds that had a high affinity for the PAR-1 receptor.

Development of secondary screening assays

We then used human platelets and human coronary artery smooth-muscle cells to establish functional assays to profile the lead compounds. Using a washed human platelet-aggregation assay to TRAP and thrombin, we demonstrated that the compounds identified in our binding assay were antagonists of PAR-1. We then showed that these lead compounds also inhibited thrombin and TRAP-stimulated calcium transients in human coronary artery smooth-muscle cells[8]. Next, we demonstrated that our compounds also inhibited TRAP and thrombin-stimulated thymidine incorporation in human coronary artery smooth-muscle cells, which is a surrogate for cellular proliferation in response to vascular injury[8]. As expected, these compounds had no effect on the enzymatic activity of thrombin.

Efficacy with PAR-1 thrombin receptor antagonists in animal models of thrombosis

The PAR-1 thrombin receptor antagonists identified in the assays described above were evaluated in various animal models. As mentioned, we were limited to the use of non-human primates because of species differences in the thrombin receptor on platelets. As our goal was to develop orally

active drugs, we established an *ex vivo* model of platelet aggregation in chair-trained conscious cynomolgus monkeys. Compounds were dosed orally, and blood samples were collected at baseline and at several time points post dosing. Utilizing a whole-blood platelet-aggregation assay and TRAP, we demonstrated the antiplatelet effects of our lead compounds. The blood samples were also used to determine the plasma concentrations of the drugs. At the end of the experiment, the animals were returned to the colony and used again after an appropriate washout period. Having demonstrated the antiplatelet effects of the PAR-1 thrombin receptor antagonists, we then evaluated lead compounds in various models of thrombosis.

In collaboration with Laurence Harker and his group at Emory University (Atlanta, Georgia, USA) we tested SCH 205831, which is a potent, selective and orally active thrombin receptor antagonist[9], in an arteriovenous-shunt model of thrombosis in conscious baboons. In this model, an exterior shunt was placed between the femoral artery and femoral vein catheters. A thrombogenic stimulus in the form of uncoated stents or a segment of a blood vessel placed in the shunt was used to initiate thrombosis. Using autologous indium-111-labelled platelets and gamma-camera imaging, platelet deposition on the surface of the stents or a segment of the blood vessel was monitored. The PAR-1 thrombin receptor antagonist SCH 205831 at an oral dose of 10 mg/kg significantly inhibited thrombosis in this model[10]. More recently, we evaluated another PAR-1 thrombin receptor antagonist, SCH 602539, in a Folts model of thrombosis in anaesthetized cynomolgus monkeys[11]. In this model, the carotid artery was isolated and a mechanical injury was performed. In addition, a flow-limiting stenosis was produced with a Lexan occluder. Formation of a thrombus was monitored by measuring blood flow. SCH 602539 inhibited thrombosis in a dose-dependent manner. We also demonstrated similar efficacy with cangrelor, which is an intravenous, reversible and direct-acting $P2Y_{12}$ ADP receptor antagonist currently in late-stage clinical development from The Medicines Company. In the same model, we also showed that the efficacy of these two agents was additive when co-administered, as SCH 602539 and cangrelor block different pathways of platelet activation (the PAR-1 thrombin receptor and the $P2Y_{12}$ ADP receptor, respectively). We have therefore demonstrated the efficacy and clinical impact of a PAR-1 thrombin receptor antagonist in animal models of thrombosis. Our findings are in agreement with published data from other groups that have shown efficacy with antibodies, and peptide or non-peptide PAR-1 thrombin receptor antagonists in animal models of thrombosis[12–14]. Our findings also suggest that PAR-1 thrombin receptor antagonists might complement and potentially improve the current treatment options in atherothrombosis.

Bleeding risk with PAR-1 thrombin receptor antagonists in animals

Although the efficacy of PAR-1 thrombin receptor antagonists in preventing thrombosis has been demonstrated in preclinical studies, the potential for increased bleeding with these agents needs to be evaluated. Interestingly, animal studies utilizing monoclonal antibody, peptide or non-peptide antagonists of the PAR-1 receptor have not shown increased bleeding at efficacious doses or higher[12–14]. These findings differ from those of antiplatelet drugs, such as aspirin and the $P2Y_{12}$ ADP receptor antagonist, which have demonstrated increased bleeding in animal models and humans. Although the reasons for the lack of bleeding with PAR-1 thrombin receptor antagonists in animals are not fully understood, thrombin-mediated platelet activation is thought to play a crucial role in propagating thrombosis and not initiating haemostasis. Indeed, von Willebrand factor and the collagen receptors GPVI and GPIa are essential in initiating platelet adhesion to the site of vascular injury during the formation of a haemostatic plug, which is then stabilized by thrombin-mediated fibrin formation. Interestingly, aspirin and clopidogrel attenuate/inhibit collagen-induced platelet aggregation, suggesting that these agents might interfere with thrombosis and haemostasis. The PAR-1 thrombin receptor antagonist therefore offers the potential to provide incremental efficacy exceeding the current standard of care without incurring an additional risk for bleeding, and this hypothesis needs to be tested clinically.

Thrombin receptor antagonists versus direct thrombin inhibitors (DTIs)

The mechanism of action of the PAR-1 thrombin receptor antagonist is distinct from those of the parenteral DTIs that are approved for clinical use (including hirudin, argatraban and bivalirudin) and the oral DTIs that are currently in late-stage clinical development (such as dabigatran)[15]. These DTIs function by binding directly to the catalytic site and the exosite 1 of the thrombin molecule in either a reversible (bivalirudin) or an

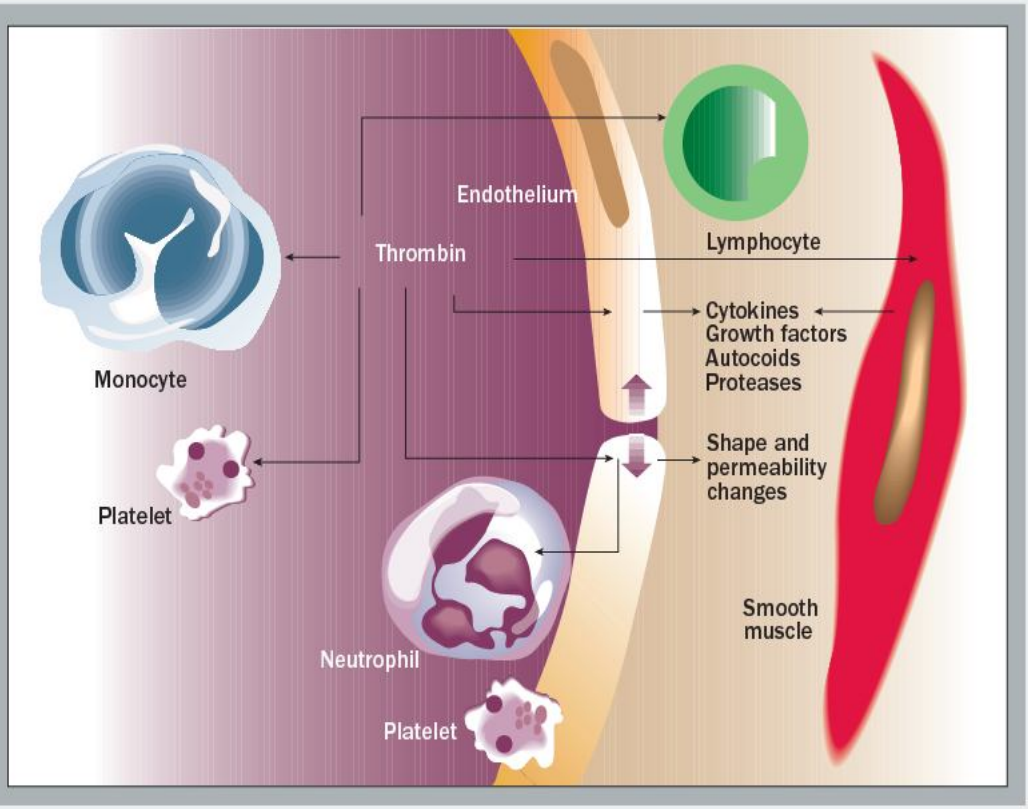

Figure 2 | Actions of thrombin on blood cells and blood vessels. Thrombin is a multifunctional serine protease generated at sites of vascular injury. It is arguably the most effective agonist for platelet activation. It also elicits a host of responses in the vascular endothelium, including shape and permeability changes, mobilization of adhesive molecules to the endothelial surface, and stimulation of autocoid (small-molecule mediators such as prostaglandins and platelet-activating factor) and cytokine production. Thrombin is also chemotactic for monocytes, and mitogenic for lymphocytes and mesenchymal cells. Reprinted with permission from ref. 20.

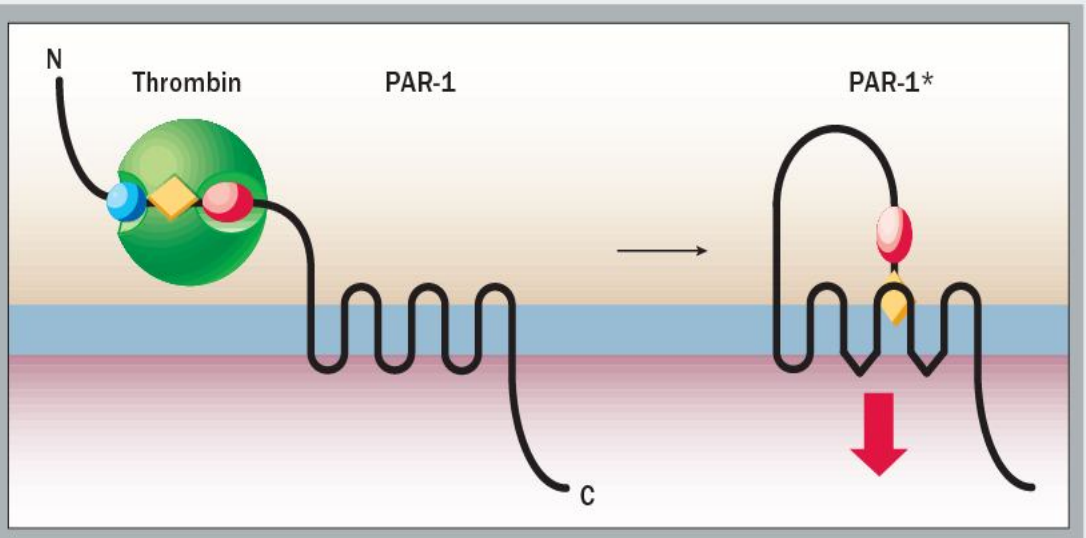

Figure 3 | Mechanism of PAR-1 activation. Thrombin (large green sphere) recognizes the amino (N)-terminal exodomain of the G-protein-coupled thrombin receptor PAR-1. This interaction uses sites that are both N-terminal (small blue sphere) and carboxy (C)-terminal (small pink oval) to the thrombin cleavage site. Thrombin cleaves the peptide bond between the receptor residues Arg41 and Ser42. This serves to unmask a new N terminus, which begins with the sequence SFLLRN (yellow diamond) that functions as a tethered ligand, docking intramolecularly with the body of the receptor to effect transmembrane signalling. Synthetic SFLLRN peptide, which mimics the tethered ligand sequence, will function as an agonist independently of receptor cleavage. PAR-1 is therefore, in essence, a peptide receptor that carries its own ligand, with the latter being active only after receptor cleavage. Reprinted with permission from ref. 20.

irreversible (hirudin) manner. By contrast, the PAR-1 thrombin receptor antagonists do not bind directly to thrombin; rather, they bind to a specific site in the transmembrane region of the PAR-1 receptor, and prevent signalling by the tethered ligand that is exposed by thrombin cleavage of the receptor. PAR-1 antagonists therefore selectively block only the receptor-mediated effects of thrombin, including those on platelets, and do not inhibit fibrin formation. The DTIs, by contrast, are primarily effective in inhibiting fibrin formation[15]. Upon direct binding, DTIs might indirectly inhibit the receptor-mediated effects of thrombin by making it unavailable for these functions. However, it should be noted that sub-nanomolar concentrations of thrombin can maximally activate platelets through the high-affinity PAR-1 receptor. It therefore remains to be seen whether DTIs can clinically interfere with thrombin-mediated platelet activation without causing profound systemic anticoagulation in patients.

Phase I clinical studies with SCH 530348

Schering-Plough has advanced the novel, potent, selective and orally active PAR-1 thrombin receptor antagonist SCH 530348 into clinical development. Studies in healthy human volunteers have demonstrated that SCH 530348 is safe and well tolerated[15]. Assessment of platelet function by an *ex vivo* platelet-aggregation assay in platelet-rich plasma demonstrated that single oral doses of SCH 530348, ranging from 5 to 40 mg, yielded >90% mean inhibition of TRAP-induced platelet aggregation across all subjects for >72 h[16]. Mean inhibition of >90% was observed at 1 h with doses of 20 and 40 mg, and at 1–2 h with a dose of 10 mg. Daily dosing with 1, 3 or 5 mg SCH 530348 for 28 days resulted in >90% mean inhibition of TRAP-induced platelet aggregation across all subjects at each dose by day 7.

Phase II clinical trial of SCH 530348

Following the completion of the initial phase I clinical studies, SCH 530348 was advanced to a phase II clinical trial in patients, known as the Thrombin Receptor Antagonist for Cardiovascular Event Reduction in Percutaneous Coronary Interventions (TRA-PCI) study. This randomized double-blind placebo-controlled dose-ranging study of loading and maintenance doses of SCH 530348 was conducted at 76 international sites, and its major findings were presented at the American College of Cardiology (ACC) i2 summit in 2007 (ref. 17). The primary objective of the TRA-PCI study was to evaluate the safety and tolerability of SCH 530348 among patients undergoing non-emergent PCI. The protocol was reviewed and approved by all relevant institutional review boards and ethics committees. Eligible subjects were those scheduled to undergo non-emergent PCI, or non-emergent coronary angiography with the intention to perform PCI if clinically indicated, for whom the elective use of an intravenous GPIIb/IIIa receptor antagonist was not planned. The TRA-PCI study randomized 1,030 patients in a 3:1 ratio to loading doses of 10, 20 or 40 mg SCH 530348 or placebo[17]. Of these, 573 patients went on to PCI and were randomized to maintenance dosing (the primary evaluable cohort). The remaining 457 patients did not undergo PCI (the secondary evaluable cohort); of these, 76 subsequently underwent coronary artery bypass grafts (CABGs). Patients in the primary cohort were further randomized to a daily maintenance dose of 0.5, 1.0 or 2.5 mg SCH 530348 or placebo (depending on the loading therapy) to complete 60 days of treatment. Patients also received other standard therapies, including aspirin, clopidogrel and an antithrombin. The primary safety end point was the incidence of thrombolysis in myocardial infarction (TIMI) major or minor bleeding at 60 days in the PCI cohort. The secondary end points included the composite of death and major adverse cardiac events (MACEs) at 60 days, again in the PCI cohort. The results from this study are summarized in Table 1. For the primary safety end point, SCH 530348 compared with placebo was not associated with a difference in the occurrence of TIMI major plus minor bleeding (2.8% versus 3.3%, respectively). When examined by loading dose treatment, TIMI major plus minor bleeding was observed in 1.6, 2.5 and 4.0% (versus 3.3% for placebo) of the patients receiving SCH 530348 doses of 10, 20 and 40 mg, respectively[17]. The major secondary efficacy outcomes for patients undergoing PCI are also summarized in Table 1. Despite the small sample size and short duration of treatment, there was a non-statistically significant but numerically smaller proportion of SCH 530348-treated versus placebo-treated patients with clinical events, which was dose related. Death/MACE was observed in 5.9% of patients treated with SCH 530348 versus 8.6% for placebo. There was also a non-statistically significant trend in the reduction of MI in the SCH 530348-treated versus placebo-treated patients (4.3% versus 7.3%, respectively). Overall, treatment with SCH 530348 reduced the incidence of arterial thrombotic events in the study population without an increase in bleeding risk. A key TRA-PCI substudy evaluated the percentage inhibition of TRAP-induced platelet aggregation. A 40-mg loading dose of SCH 530348 achieved >80% inhibition of platelet aggregation in 1–2 h for 68–96% of subjects, and maintenance doses of 1 mg and 2.5 mg led to

Table 1 | TRA-PCI results

	SCH 530348				
	Placebo	All doses	10 mg	20 mg	40 mg
	(*n* = 151)	(*n* = 422)	(*n* = 129)	(*n* = 120)	(*n* = 173)
TIMI major/minor bleeding*	5 (3.3%)	12 (2.8%)	2 (1.6%)	3 (2.5%)	7 (4.0%)
Non-TIMI bleeding	48 (32%)	173 (41%)	47 (36%)	52 (43%)	74 (43%)
Death/MACE†	13 (8.6%)	25 (5.9%)	11 (8.5%)	6 (5.0%)	8 (4.6%)
MI	11 (7.3%)	18 (4.3%)	7 (5.4%)	5 (4.2%)	6 (3.5%)

*Primary end point (safety). †Secondary end point (efficacy). MACE, major adverse cardiac event; MI, myocardial infarction; PCI, percutaneous coronary intervention; TIMI, thrombolysis in myocardial infarction.

sustained >80% inhibition of platelet aggregation at 30 and 60 days in all subjects. Based on the encouraging results of the TRA-PCI study, Schering-Plough has advanced SCH 530348 to two large phase III clinical trials designed to evaluate its efficacy and safety in patients with acute coronary syndromes (TRA*CER; *n* = 10,000) and in the setting of secondary prevention (TRA*2P TIMI 50; *n* = 19,500).

Potential utility of PAR-1 thrombin receptor antagonists in other clinical indications including the treatment of restenosis and inflammatory diseases

The PAR-1 thrombin receptor is also expressed on a host of inflammatory cells and constituents of the vessel wall (Fig. 2), and mediates proinflammatory, chemotactic and mitogenic responses. The expression of the PAR-1 thrombin receptor is upregulated in human atherosclerotic lesions and in experimental models of restenosis in animals. Small-molecule PAR-1 thrombin receptor antagonists have been reported to inhibit restenosis experimentally. The expression of PAR-1 thrombin receptors is also upregulated in human inflammatory bowel disease, and PAR-1 antagonists have demonstrated benefits in mouse models of inflammation[18]. Furthermore, there is a growing body of experimental evidence to show that PAR-1 thrombin receptors are upregulated in radiation enteropathy, pulmonary hypertension, ischaemia/reperfusion injury and other pathological conditions. PAR-1 thrombin receptor antagonists therefore have potential utility in the treatment of a broad range of diseases, which needs to be explored clinically.

Schering-Plough's contribution to the field of atherothrombosis

For more than two decades, Schering-Plough has supported research aimed at the reduction of risk factors for atherothrombosis[3,4,10,11,19]. These activities have contributed to a more complete understanding of the pathophysiological processes that are involved in the disease, and to the development of effective new products with excellent safety profiles.

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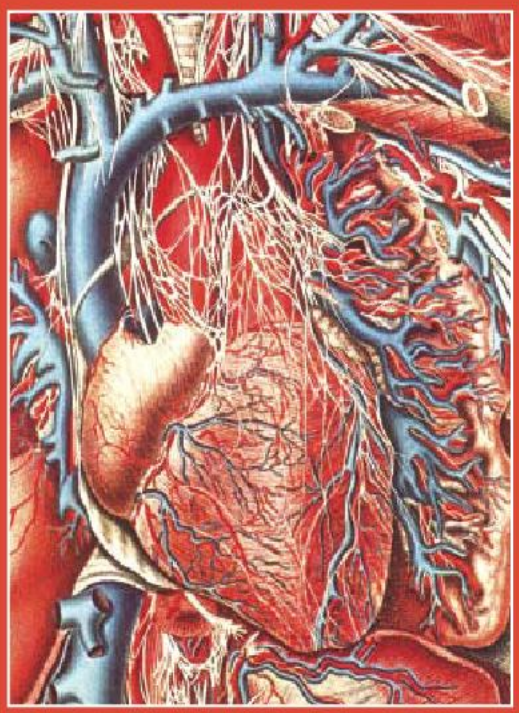

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ARTICLES

A photosynthetic alveolate closely related to apicomplexan parasites

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Many parasitic Apicomplexa, such as *Plasmodium falciparum*, contain an unpigmented chloroplast remnant termed the apicoplast, which is a target for malaria treatment. However, no close relative of apicomplexans with a functional photosynthetic plastid has yet been described. Here we describe a newly cultured organism that has ultrastructural features typical for alveolates, is phylogenetically related to apicomplexans, and contains a photosynthetic plastid. The plastid is surrounded by four membranes, is pigmented by chlorophyll *a*, and uses the codon UGA to encode tryptophan in the *psbA* gene. This genetic feature has been found only in coccidian apicoplasts and various mitochondria. The UGA-Trp codon and phylogenies of plastid and nuclear ribosomal RNA genes indicate that the organism is the closest known photosynthetic relative to apicomplexan parasites and that its plastid shares an origin with the apicoplasts. The discovery of this organism provides a powerful model with which to study the evolution of parasitism in Apicomplexa.

Alveolata Cavalier-Smith, 1991 (emended by ref. 1)
Chromerida phyl. nov.
Chromera velia gen. et sp. nov.

Etymology. *Chromera* (feminine), derived from the English words chromophore and meront, because in pure culture the pigmented plastid was inherited through cell division; *velia* (feminine), a modern Italian proper name, meaning veiled or concealed (Supplementary Information).

Holotype/hapantotype. Z.6967 (Australian Museum, Sydney), preserved culture embedded in PolyBed 812 (electron micrographs shown in Fig. 1a, b) and separately in absolute ethanol. The culture is NQAIF136 (North Queensland Algal Culture and Identification Facility, James Cook University, Townsville, Australia). The clonal culture consists of dividing immotile organisms. Culture submission date, 25 February 2004; culture isolation date, 13 December 2001; isolator, R.B.M.

Locality. Scleractinian coral *Plesiastrea versipora* (type host; Lamarck, 1816) (Metazoa: Cnidaria: Faviidae) obtained from Sydney Harbour, New South Wales, Australia (latitude 33° 50′ 38.76″ S; longitude 151° 16′ 44″ E) at ~3-m depth. Collection date, 7 December 2001. Collectors: T. Starke-Peterkovic and L. Edwards.

Referred material. Additional cultures are CCAP 1602/1 (Culture Collection of Algae and Protozoa, Scottish Association of Marine Science, UK) and CCMP2878 (Provasoli-Guillard Center for Culture of Marine Phytoplankton, Maine, USA).

Diagnosis. Unicellular (Fig. 1a). The immotile life stage reproduces by binary division (Fig. 1b), but is not restricted to binary division. The immotile life stage is spherical to sub-spherical, 5–7 μm in diameter in the G1 phase of the cell cycle. Cell diameter is up to 9.5 μm in other cell cycle phases. A golden-brown, cone-shaped plastid is present. Immediately after completion of binary division, nascent cells contain a single plastid only. Thylakoid lamellae are in stacks of three or more (Fig. 1c). The plastid is bounded by four membranes (Fig. 1d) and contains chlorophyll *a*, but no other chlorophylls. A micropore is present (Fig. 1c). Internal cilium/cilia are present and anchored at the cell periphery, extending to the periplastidal compartment (Fig. 1a, f, g). Cortical alveoli are flattened, with underlying microtubules (Fig. 1e). The position of attachment of internal cilium to the cell periphery is defined as the apex of the immotile cell. There is a single, large mitochondrion ~1 μm in diameter (Fig. 1a). Mitochondrial cristae are lamellar, ampulliform and tubular in structure. Vesicles of diameter ~1 μm attach to and surround the large mitochondrion. The cell wall surface is smooth, with a raised ridge ~85 nm wide, extending around an incomplete circle and forking periodically (Supplementary Information). *Chromera velia* is free-living or associated with stony corals; it is the type species of the phylum Chromerida. Chromerida differ from all known alveolates[1] (Supplementary Information) in having a photosynthetic secondary plastid bearing chlorophyll *a*, but no chlorophyll *c*[2].

Plastids of alveolates and their medical significance

The alveolates are a major lineage of protists that are defined by the possession of subsurface alveoli (flattened membrane-bound vesicles) supported by microtubules, as well as the presence of micropores and mitochondria with ampulliform or tubular cristae[1,3]. Alveolates are divided into three main phyla: the ciliates, apicomplexans and dinoflagellates. The group Apicomplexa[1] (Levine, 1970; emended by Adl *et al.* 2005 (ref. 1)) is composed mostly of parasites that are united by the possession of a set of secretory organelles

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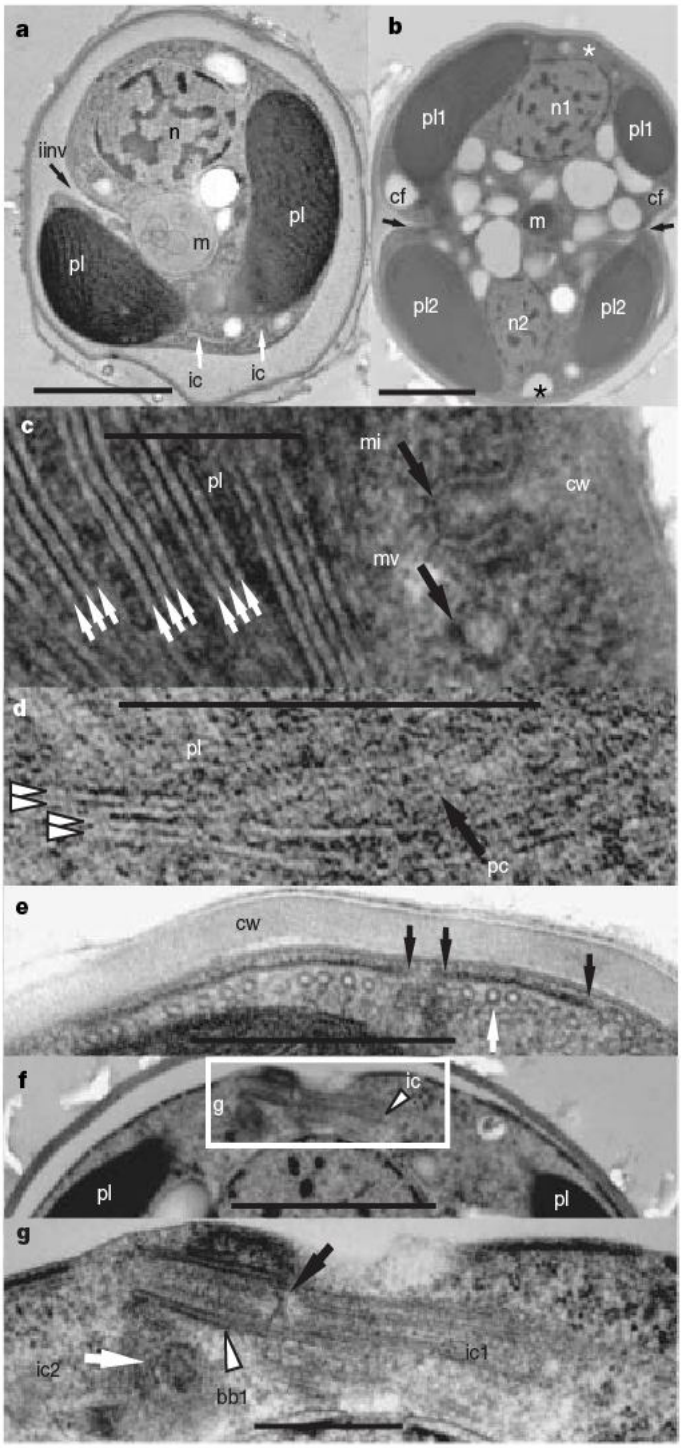


Figure 1 | Ultrastructure of new alveolate *Chromera velia*. **a**, Interphase (scale bar, 2 μm). iinv, interphase invagination (black arrow); m, mitochondrion; n, nucleus; pl, plastid. **b**, Binary division. The apex of each daughter cell is marked by an asterisk. The mitochondrion of the mother cell is centrally positioned at the terminator of the cleavage furrow, between the nuclei of daughter cells 1 and 2 (scale bar, 2 μm). cf, cleavage furrow. **c**, Micropore (mi) and thylakoid lamellae. The micropore is an invagination of the plasma membrane. An associated vesicle in the cytoplasm is indicated (mv, micropore-associated vesicle). Thylakoid lamellae in the plastid are in sets of three (white arrows) or more (scale bar, 200 nm). cw, cell wall. **d**, The two pairs of plastid membranes separate at the periplastidal compartment (white triangles). The outer pair forms the periplastidal compartment (scale bar, 200 nm). pc, periplastidal compartment. **e**, Alveoli and supporting microtubules. Alveoli lying beneath the plasma membrane abut each other closely (at black arrows) and are underlain by microtubules (white arrow) (scale bar, 500 nm). **f**, Maintenance of the plastid (pl) in a cone shape is aided by one or more internal cilia (ic, white triangle) anchored at the apex of the cell (scale bar, 2 μm). **g**, Magnification of boxed area in **f**. Internal cilium ic1 extends inward from basal body bb1 (white triangle), which is attached to the cell periphery. ic1 and bb1 join at a terminal plate (black arrow). ic2 (white arrow) emerges perpendicular to ic1 (scale bar, 500 nm). **a**, **b**, Hapantotype Z.6967 (Australian Museum, Sydney).

underlying an oral structure at the anterior apex of the cell[4] (the 'apical complex'), and other characters. Within the phylum is a monophyletic subgroup of obligate parasites that comprises some 6,000 taxa[5]. These present a major burden to livestock and human health. Many contain a relic plastid termed the apicoplast[6]. Among the apicomplexans, it is specifically hemosporidians, piroplasms (both groups are blood parasites, including *Plasmodium*, that cause malaria) and coccidians (for example, *Toxoplasma gondii*[7] and the veterinary pathogen *Eimeria*) that possess an apicoplast. Because animals do not possess plastids, the apicoplast represents an opportunity to target these parasites with treatments that are relatively harmless to mammalian hosts[8].

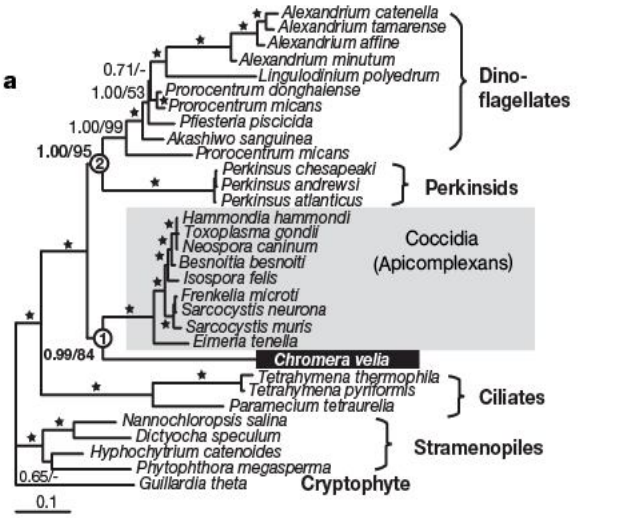
The reduced 35-kilobase genome and imported proteome of the *Plasmodium* apicoplast have been exhaustively studied. Several critical pathways are localized in the apicoplast, including fatty acid and isoprenoid biosynthesis[6]. However, not all apicomplexans possess this organelle. No evidence of a plastid has been found in *Gregarina*, the only gregarine examined so far[9]. Similarly, the waterborne parasite *Cryptosporidium* lacks an apicoplast[10,11]. Finally, there is no published evidence for apicoplasts in colpodellids, a group of non-parasitic apicomplexans that possess an apical complex of organelles used for predation on algal and protist prey[4,12].

In the absence of an extant alga that represents the ancestral photosynthetic state of these diverse apicomplexans, the evolutionary descent of the apicoplast has instead been indicated by taxonomic and phylogenetic affiliation of apicomplexans to particular algae. Gene phylogenies relate the apicoplasts to the chloroplasts of a subset of dinoflagellate algae—those that are pigmented by the chromophore peridinin[13,14]. It is thought that the common ancestor of peridinin dinoflagellates and apicomplexans possessed a chromalveolate plastid containing the specific combination of chlorophyll *a* and chlorophyll *c*[13,15]. Whereas peridinin dinoflagellates retained the plastid, degeneration of the photosynthetic chromalveolate plastid occurred independently in many other dinoflagellates and also occurred independently in apicomplexans[16]. In a range of dinoflagellates, photosynthetic plastids were ingested and replaced the chromalveolate plastid[16]. In other dinoflagellates, the chromalveolate plastid was lost and not replaced, resulting in heterotrophy[4,15,17]. In parasitic apicomplexans the plastid remains, but in a non-photosynthetic form. Here we show that *C. velia* is a relative of parasitic apicomplexans and colpodellids, and bears a photosynthetic plastid that is related most closely to apicoplasts but also to peridinin dinoflagellate plastids, affirming a common ancestry. *Chromera velia* can live independently of its host and is easily maintained in culture. As well as providing a model to study apicomplexan evolution, we predict that *C. velia* will be of practical use in high-throughput screening of prospective anti-apicoplast drugs.

Evolutionary position of *Chromera velia*

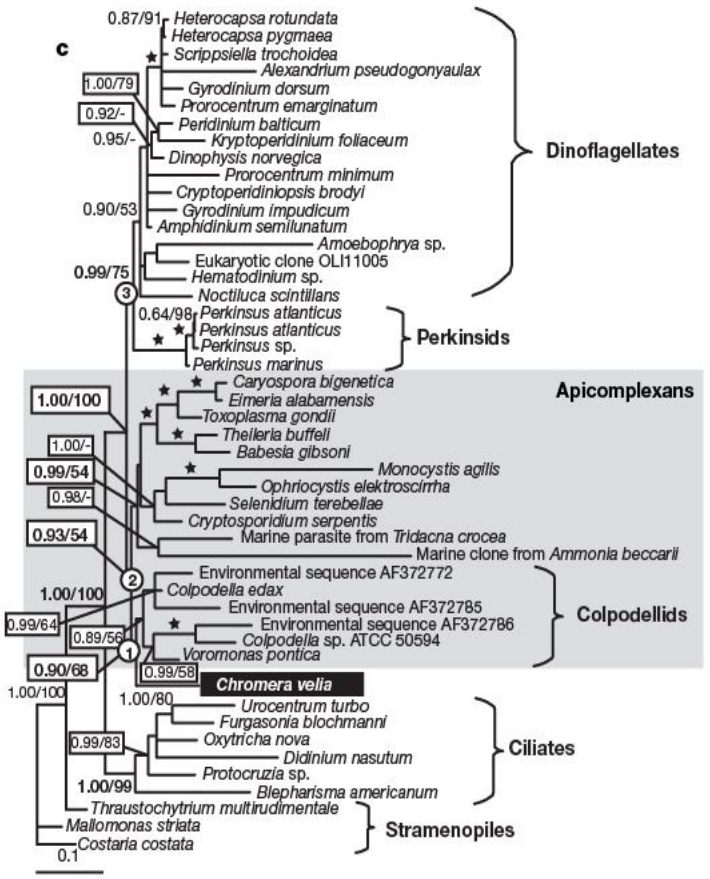
The three ultrastructural features diagnostic of alveolates[1,3] are all present in *C. velia*: first, a micropore occurs in the cell periphery (Fig. 1c); second, subsurface alveoli are present and supported by microtubules (Fig. 1e); and third, the mitochondrion (Fig. 1a) contains ampulliform and tubular cristae (Supplementary Information).

Molecular phylogenetic analyses of nuclear genes showed that *C. velia* is more closely related to apicomplexan parasites than to photosynthetic dinoflagellates. In bayesian and maximum likelihood analyses of nuclear large subunit (LSU) rDNA sequences, *C. velia* branched at the root of the Apicomplexa (Fig. 2a), with this position corroborated by a topology test (Fig. 2b) and by slow–fast analysis (Supplementary Information). Phylogeny of nuclear small subunit (SSU) rDNA sequences also supported a close relationship between *C. velia* and apicomplexans, with the new taxon branching at the root of colpodellids (Fig. 2c). Although the position of *Chromera* on this tree had relatively low bootstrap and posterior probability support (maximum likelihood bootstrap 68, posterior probability 0.90),



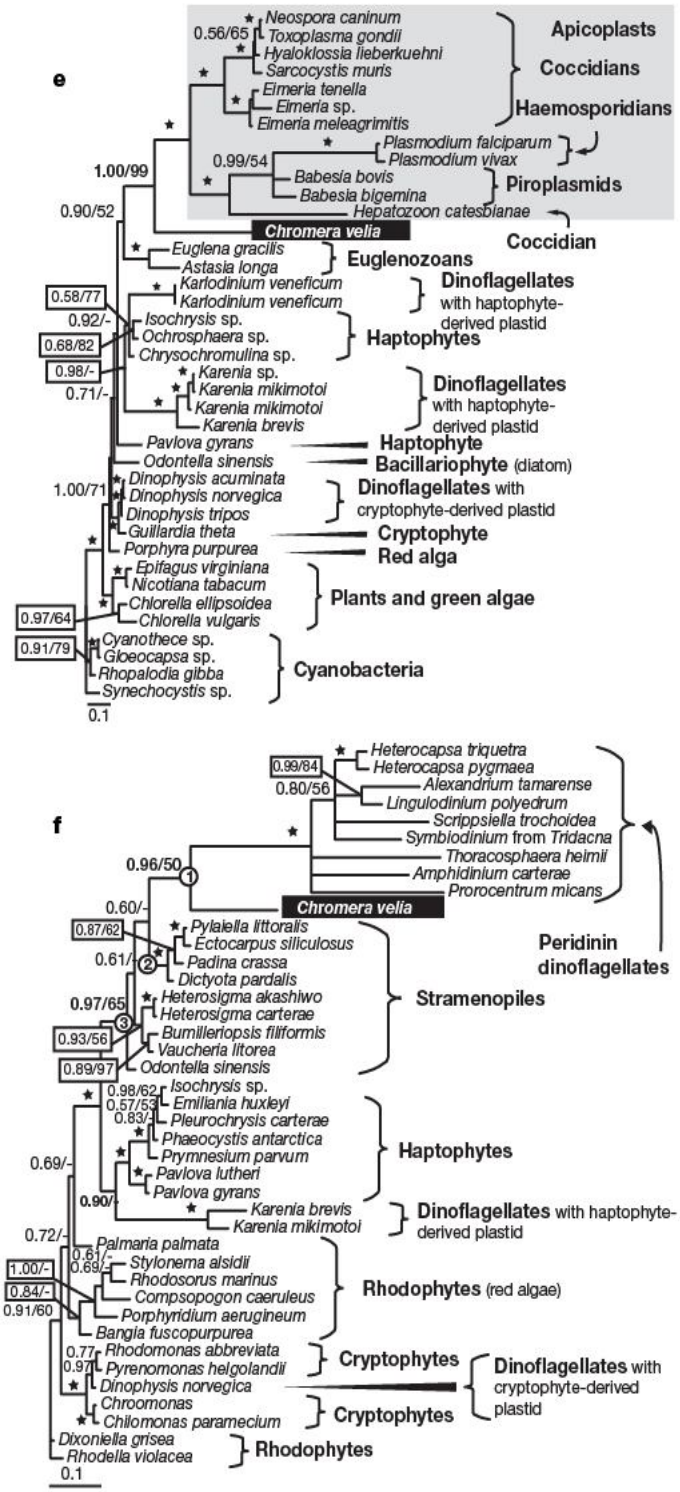
b

Nuclear LSU rDNA	au	np	bp	pp	kh	sh	wkh	wsh
Topology 1	0.989	0.991	0.990	1.00	0.988	0.988	0.988	0.988
Topology 2	0.011	0.009	0.010	$9x10^{-13}$	0.012	0.012	0.012	0.012



d

Nuclear SSU rDNA	au	np	bp	pp	kh	sh	wkh	wsh
Topology 1	0.955	0.634	0.631	0.709	0.743	0.856	0.743	0.901
Topology 2	0.150	0.351	0.358	0.291	0.257	0.610	0.257	0.491
Topology 3	0.006	0.011	0.011	$2x10^{-6}$	0.039	0.044	0.039	0.046



g

PsbA	au	np	bp	pp	kh	sh	wkh	wsh
Topology 1	0.736	0.699	0.698	0.972	0.705	0.843	0.705	0.808
Topology 2	0.334	0.285	0.289	0.027	0.295	0.368	0.295	0.416
Topology 3	0.041	0.016	0.013	0.001	0.074	0.144	0.074	0.144

Figure 2 | Nuclear and plastid phylogenies of *Chromera velia*. **a**, Bayesian phylogenetic tree of nuclear LSU rDNA inferred from 2,740 characters (GenBank accession EU106870). **b**, Topology tests of the placement of the *C. velia* branch with respect to branches of the nuclear LSU rDNA tree. Numbered branches are indicated in **a**. Topology test results are: the *P*-value for the approximately unbiased test (au) calculated from the multiscale bootstrap; the non-parametric bootstrap probability calculated from the multiscale bootstrap (np); the bootstrap probability calculated in the non-multiscale manner (bp); the bayesian posterior probability calculated by the bayesian-information-criterion approximation (pp); and the *P*-values of the Kishino–Hasegawa test (kh), the Shimodaira–Hasegawa test (sh), the weighted Kishino–Hasegawa test (skh) and the weighted Shimodaira–Hasegawa test (wsh). **c**, Bayesian phylogenetic tree of nuclear SSU rDNA inferred from 1,285 characters (GenBank accession DQ174732). **d**, Topology tests of the placement of the *C. velia* branch with respect to branches of the nuclear SSU rDNA tree. Numbered branches are indicated in **c**. **e**, Bayesian phylogenetic tree of plastid SSU rDNA gene sequences inferred from 811 characters (GenBank accession EU106871). **f**, Bayesian phylogenetic tree of the PsbA photosynthesis protein inferred from 319 characters (GenBank accession EU106869). **g**, Topology tests of the placement of the *C. velia* branch with respect to branches of the PsbA tree. Numbered branches are indicated in **f**. On all trees, black stars indicate branches with bayesian posterior probabilities higher than 0.99 and maximum likelihood bootstrap support higher than 90%.

topology tests rejected alternative placement of *Chromera* at the root of dinozoans (Fig. 2d).

The lineage of the *C. velia* plastid was analysed using plastid rDNA and PsbA, a plastid protein that is part of photosystem II. In the phylogenetic analysis of plastid SSU rDNA, the *C. velia* chloroplast branched at the root of the apicoplasts with good support (Fig. 2e). It was not possible to include sequences from the peridinin-pigmented plastids of dinoflagellates in the plastid SSU rDNA analyses because their high divergence caused their position in trees to be unstable. The PsbA sequence of *C. velia* was found to be conserved when compared with that of other photosynthetic eukaryotes (Supplementary Information), and phylogenetic analysis of PsbA was therefore restricted to relevant taxa to avoid the effects of homoplasy across unrelated lineages. Taxa were selected based on the strong relationships shown between *C. velia*, dinoflagellates and stramenopiles on the nuclear rDNA trees. The selected set included dinoflagellates, other chromalveolates and rhodophytes (Fig. 2f). Apicoplasts could not be included as they do not possess the *psbA* gene. Although maximum likelihood support across the PsbA protein tree was limited, there was significant bayesian support for known relationships, such as a grouping of stramenopile and dinoflagellate secondary plastids (posterior probability 0.97, maximum likelihood 65), and a grouping of haptophyte secondary plastids as neighbour to these two (posterior probability 0.99, maximum likelihood 90). The analysis supported *C. velia* as a relative of peridinin dinoflagellate plastids (posterior probability 0.96, maximum likelihood 50). Topology testing (Fig. 2g) corroborated the most likely placement of the *C. velia* plastid as closest sister group to the plastids of peridinin dinoflagellates, as was expected given that apicomplexans were not included in the analysis.

Unique features of the *Chromera velia* plastid

The *psbA* gene of *C. velia* contains an unusual codon that links the plastid to the apicoplast lineage. All other eukaryotic algae use UGG codons to encode tryptophan at seven conserved positions in the gene. The *C. velia* gene instead uses UGA codons at these positions (Supplementary Information). The UGA-Trp codon is unprecedented in any photosynthetic plastid and has only been found in the apicoplasts of coccidians and in various mitochondria[6,18].

The *C. velia* plastid contains thylakoid lamellae in stacks of three or more (Fig. 1c), resembling the arrangement in the plastids of peridinin-pigmented dinoflagellates[19]. It displays novel pigmentation, with chlorophyll *a*, violaxanthin and a novel carotenoid as the major components, and β,β-carotene as a minor component (Supplementary Information). No other chlorophylls are present. Mass spectrometry analysis indicated that the novel carotenoid is an isomer of isofucoxanthin (Supplementary Information). This finding is consistent with the *Chromera* plastid being red-derived, as isomers of isofucoxanthin are absent from plastids of the green lineage[2]. Pulse amplitude modulation fluorescence analysis confirmed that photosynthesis occurs in *Chromera* (Supplementary Information). Assuming that the apicomplexan–dinoflagellate group was ancestrally photosynthetic, the absence of chlorophyll *c* in *C. velia* was unexpected, as peridinin dinoflagellates normally possess this pigment. We propose that secondary loss of chlorophyll *c* could have occurred in early apicomplexans.

An ultrastructural feature in common between the *C. velia* plastid and apicoplasts is the number of surrounding membranes. It is generally presumed that the number of membranes surrounding a stable plastid can decrease but not increase during its evolutionary specialization[20]. The *C. velia* plastid is surrounded by four membranes (Fig. 1d). Reports vary in their estimates of the number of membranes surrounding apicomplexan plastids. Three-dimensional reconstruction of the *P. falciparum* apicoplast indicated three membranes[21], supplemented with additional inner and outer membrane complexes. A similar reconstruction of the apicoplast of the coccidian *T. gondii* found spatial alternation of two and four membranes[22]. By comparison, the plastids of peridinin dinoflagellates are surrounded by three membranes[19,20]. We suggest that the four membranes bounding the *C. velia* plastid may represent the number surrounding the ancestor of apicoplasts and peridinin dinoflagellate plastids.

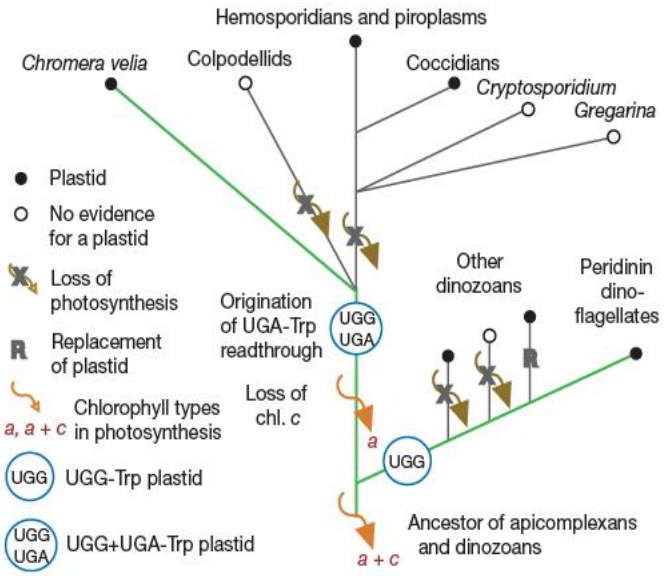


Figure 3 | Evolution of *Chromera velia*, apicomplexans and dinoflagellates. The path in green traces the maintenance of photosynthesis. Characteristics of the terminal nodes of coccidia, hemosporidians and piroplasms are generalized. The gregarine shown is *Gregarina niphandroides*[9]. The *Cryptosporidium* species represented is *C. parvum*[11,25]. 'Other dinozoans' includes non-photosynthetic species: *Perkinsus atlanticus* (filled black circle, plastid present[26]) and *Oxyrrhis marina* (open circle, no plastid evident[27]). The dinozoan branch bearing 'replaced plastids' is a symbolic branch representing many such branches that obtained tertiary plastids independently. Heterotrophic dinoflagellates have characters as for *Oxyrrhis marina*. The tree is a consensus of data presented in this paper and other published relationships[10,12,13,28–30].

Concluding remarks

Phylogenetic analyses support the description of *Chromera velia* as an alveolate, possessing a photosynthetic plastid that lies in the same secondary endosymbiotic lineage as apicoplasts. The ultrastructure and photosynthetic pigment profile of *C. velia* are consistent with a chromalveolate-affiliated ancestry. Figure 3 presents a model of the evolutionary history of *C. velia*, apicomplexans and dinoflagellates based on the phylogeny of the nuclear and plastid lineages and the retention or loss of plastid characteristics. *Chromera velia* represents the closest known photosynthetic relative of apicomplexan parasites.

METHODS SUMMARY

Chromera velia was isolated from the stony coral *Plesiastrea versipora* (Faviidae) from Sydney Harbour (Australia) by a variation of a procedure[23] normally used to isolate intracellular symbionts of the genus *Symbiodinium* (Supplementary Information). Unicellular lines were generated by streaking raw colonies onto an agar-gelled minimal growth medium, picking single colonies and regrowing in liquid medium (Supplementary Information). Genomic DNA was extracted and genes (nuclear SSU and LSU rDNA, and plastid SSU rDNA and *psbA*) were amplified and sequenced. Purity of cultures was checked by sequencing multiple nuclear SSU rDNA clones from each unialgal line (Supplementary Information). Sequences were aligned, and phylogenetic analyses were performed using maximum likelihood and bayesian inference. Selected data sets were analysed using a slow–fast method. Specimens for transmission electron microscopy (TEM) were prepared using a freeze-substitution method[24] and examined by TEM. Scanning electron microscopy (SEM) specimens were prepared using standard methods (Supplementary Information). Pigments were extracted and analysed by a combination of high-performance liquid chromatography (HPLC), ultraviolet and visible (UV/Vis) spectra analysis and mass spectrometry (MS), and were identified by comparison of their retention times and spectra to those of mixed standards obtained from known cultures (Supplementary Information).

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Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

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Author Contributions R.B.M. isolated the strain while in the D.A.C. laboratory, then while in the J.M.L. laboratory designed the AToL SSU primers and the *psbA* primers, cloned and sequenced multiple copies of the SSU rRNA gene, a copy of the plastid SSU rRNA gene and initial sections of the *psbA* and LSU rRNA genes, then assigned precedented methods for culture fixation, and wrote and finalized the draft of the paper; M.O. led and performed phylogenetic analyses of the sequence data, cloned and sequenced a copy of the plastid SSU rDNA gene using different primers than R.B.M., and co-wrote the draft of the paper; M.O. and M.V. performed the TEM and SEM data collection; J.J. and T.C. cloned and sequenced near full-length LSU rDNA and *psbA* genes and undertook extensive phylogenetic analysis; T.C. performed mito-red staining; S.W.W. and N.W.D. performed pigment analysis and interpreted pigment data; R.B.M., K.H., C.J.S.B. and J.S. interpreted TEM data and assigned taxonomy; K.H. and R.B.M. performed light microscopy; R.B.M., M.O., T.C., K.H., D.H.G. and C.J.S.B. maintained cultures. D.H.G. cloned and sequenced the LSU rRNA gene, using different PCR primers than T.C. and J.J.; R.B.M., M.O., D.H.G., K.H., J.S., O.H.-G., J.M.L., C.J.S.B. and D.A.C. designed research, interpreted evolutionary, ecological and microbiological data, and performed extensive editing and revision.

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ARTICLES

Phosphoinositide signalling links *O*-GlcNAc transferase to insulin resistance

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Glucose flux through the hexosamine biosynthetic pathway leads to the post-translational modification of cytoplasmic and nuclear proteins by *O*-linked β-*N*-acetylglucosamine (*O*-GlcNAc). This tandem system serves as a nutrient sensor to couple systemic metabolic status to cellular regulation of signal transduction, transcription, and protein degradation. Here we show that *O*-GlcNAc transferase (OGT) harbours a previously unrecognized type of phosphoinositide-binding domain. After induction with insulin, phosphatidylinositol 3,4,5-trisphosphate recruits OGT from the nucleus to the plasma membrane, where the enzyme catalyses dynamic modification of the insulin signalling pathway by *O*-GlcNAc. This results in the alteration in phosphorylation of key signalling molecules and the attenuation of insulin signal transduction. Hepatic overexpression of OGT impairs the expression of insulin-responsive genes and causes insulin resistance and dyslipidaemia. These findings identify a molecular mechanism by which nutritional cues regulate insulin signalling through *O*-GlcNAc, and underscore the contribution of this modification to the aetiology of insulin resistance and type 2 diabetes.

The relentless progression of diabetes mellitus is rapidly becoming one of the principal threats to human health in the twenty-first century[1]. Nutrient excess and sedentary lifestyle are two of the major culprits fuelling the diabetes epidemic[2,3]. Type 2 diabetes results from a disruption of normal glucose homeostasis, primarily as a result of decreased peripheral insulin action coupled with relative insulin insufficiency[4,5]. However, the mechanisms by which excessive nutrients produce peripheral insulin resistance are not well understood.

Phosphatidylinositol 3,4,5-trisphosphate ($PI(3,4,5)P_3$) is a central mediator of insulin signalling[6–8]. On binding insulin, the insulin receptor (IR) catalyses tyrosine phosphorylation of the insulin receptor substrate (IRS) proteins, which results in the recruitment and activation of phosphatidylinositol-3-OH kinase (PI(3)K). The lipid product of PI(3)K, $PI(3,4,5)P_3$, recruits a subset of signalling proteins with pleckstrin homology (PH) domains, such as phosphoinositide-dependent kinase 1 (PDK1) and Akt, to the plasma membrane, where PDK1 induces the threonine phosphorylation and activation of Akt. Together these kinases initiate complex sets of transcriptional and post-transcriptional events that promote the synthesis and storage of carbohydrates, lipids and proteins and inhibit their degradation and release into the circulation[5].

Because sustained insulin action would be detrimental to physiological homeostasis, several feedback mechanisms have evolved to attenuate signalling[9]. Protein tyrosine phosphatases and phosphoinositide phosphatases exert inhibitory effects at defined sites in the proximal insulin signalling pathway[10,11]. Phosphorylation of IRS proteins by serine/threonine kinases is emerging as a mechanism for negative-feedback inhibition of insulin signalling and for cross-talk from other signalling pathways[12,13]. This is relevant because aberrant serine phosphorylation of IRS proteins is tightly linked to the aetiology of insulin resistance[13].

Glucose flux through the hexosamine biosynthetic pathway leads to the post-translational modification of cytoplasmic and nuclear proteins by *O*-GlcNAc[14,15]. OGT catalyses the attachment of *O*-GlcNAc to proteins, whereas *O*-GlcNAcase catalyses the sugar removal[16–18]. This dynamic and reversible modification is emerging as a key regulator of various cellular processes, such as signal transduction, transcription and proteasomal degradation[15,19–22]. Perturbations in protein *O*-GlcNAc modification are implicated in various human diseases including diabetes mellitus, neurodegeneration and cancer[23–31].

The end product of hexosamine biosynthesis, UDP-GlcNAc, donates the GlcNAc moiety for this modification[32]. The UDP-GlcNAc levels fluctuate with the availability of glucose, non-esterified fatty acids, uridine and the amino acid glutamine[15,33–35]. It has therefore been proposed that *O*-GlcNAc may serve as a nutrient sensor[36,37]. Our previous studies have defined nuclear *O*-GlcNAc as a negative regulator of transcription in response to steroid hormone signalling[20,38]. To gain insight into how cytoplasmic *O*-GlcNAc couples systemic metabolic status to the regulation of signal transduction, we explored the molecular basis on which *O*-GlcNAc regulates insulin signalling in response to glucose flux, and to understand the contribution of this nutrient sensor to the aetiology of insulin resistance.

OGT interacts with phosphoinositides

Because OGT shares homology with protein phosphatase 5, which exhibits affinity for lipids[39], we examined whether OGT could interact with lipids to act as an atypical lipid sensor. In a protein–lipid overlay assay, we found that OGT has affinity for a variety of PIP species (Fig. 1a). Deletion analysis mapped the PIP-binding region within amino-acid residues 958–1001 adjacent to the catalytic domain II at the carboxy terminus of OGT (Supplementary Fig. 1). In the blots with serial dilutions of diverse phospholipids, the full-length OGT binds most tightly with $PI(3,4,5)P_3$, whereas the deletion mutants (471C and 821C) that retain the C-terminal regions also

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show strong interaction with PI(4)P and PI(3,5)P_2, indicating that the N-terminal region renders OGT specific for PI(3,4,5)P_3 (Fig. 1b). To further characterize the PIP-binding domain in OGT, its primary sequence was interrogated against public databases including PROSITE, BLOCKS, ProDom, PRINTS, Pfam and SMART. We detected no homology between OGT and any known PIP-binding motifs. We therefore named this new class of motif the PPO (PIP-binding activity of OGT) domain.

Because acidic phosphate groups in PIPs are required for the interaction with OGT (Fig. 1a), we tested whether basic residues abundant in the PPO domain mediate this protein–lipid interaction. As shown in Fig. 1c and Supplementary Fig. 2, a double mutation of lysine residues to alanines (K981A/K982A) completely abolished the binding of the OGT(919C) fragment to PIPs, and K986A and K989A single mutations decreased lipid binding. In contrast, arginines 984 and 991 and lysine 1000 were dispensable for the PIP interaction (Supplementary Fig. 2). Thus, a cluster of lysine residues in the PPO domain are involved in the interaction with PIPs.

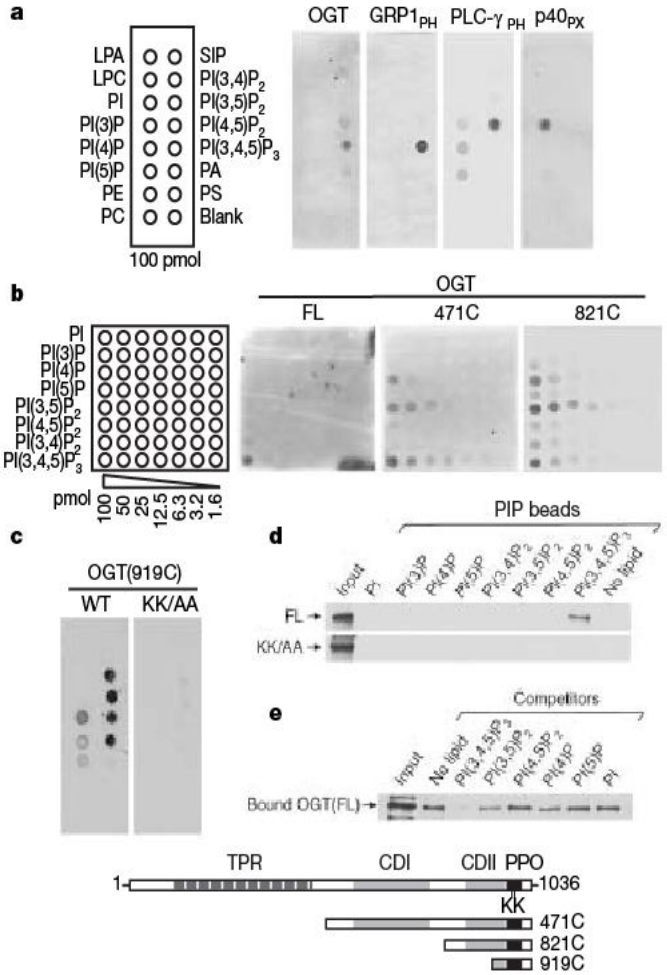

Figure 1 | OGT interacts with phosphoinositides. **a**, Binding of OGT to phospholipids immobilized on nitrocellulose membranes (PIP strips; Echelon Biosciences). Binding of the PH, PH and PX domains of GRP1, phospholipase Cγ (PLC-γ) and p40, respectively, to PIP strip lipid blots served as quality controls. Left, diagram of phospholipid species. LPA, lysophosphatidic acid; LPC, lysophosphatidylcholine; PE, phosphatidylethanolamine; PC, phosphatidylcholine; SIP, sphingosine 1-phosphate; PA, phosphatidic acid; PS, phosphatidylserine. **b**, Binding of full-length (FL) OGT and deletion mutants to lipid blots (PIP arrays; Echelon Biosciences). Left, diagram of phosphoinositide species and concentrations. **c**, Binding of OGT(919–1036) fragment to a lipid blot in the absence or presence of the KK/AA mutation. **d**, Pulling-down of full-length OGT without or with the KK/AA mutation by various phosphoinositide-coupled affinity beads (Echelon Biosciences). **e**, Pulling-down of full-length OGT by PI(3,4,5)P_3-coupled beads in the presence of various free phosphoinositides. Bottom, schematic representation of full-length OGT and various deletion mutants. TPR, tetratricopeptide repeats; CDI and CDII, catalytic domains I and II; PPO, PIP-binding domain.

We validated these results by an independent method for detecting protein–lipid interactions. Among different PIP-coupled affinity beads, the full-length OGT bound specifically to beads bearing PI(3,4,5)P_3, which was abolished by the (K981A/K982A) mutation (Fig. 1d). The addition of free PI(3,4,5)P_3 effectively displaced OGT from PI(3,4,5)P_3 beads, but other free PIPs had no effect (Fig. 1e). These results confirmed that OGT can bind selectively to PI(3,4,5)P_3 through the PPO domain.

PI(3,4,5)P_3 mediates OGT translocation

The ability of OGT to bind PI(3,4,5)P_3 *in vitro* raises the question of whether they functionally interact *in vivo*. In an OGT activity assay, none of the PIP species measurably affected the ability of OGT to modify a protein substrate, p62, arguing against a role for PIPs in regulating the catalytic activity of the enzyme (Supplementary Fig. 4).

Activation of PI(3)K leads to the accumulation of PI(3,4,5)P_3 at the plasma membrane in response to various extracellular signals such as insulin. The interaction between OGT and PI(3,4,5)P_3 prompted us to examine the subcellular localization of OGT on PI(3)K signalling. As shown in Fig. 2a and Supplementary Movies 1 and 3, green fluorescent protein (GFP)–OGT translocated rapidly to the plasma membrane within 90 s of stimulation with serum in live cells. Pretreatment with the PI(3)K inhibitor wortmannin completely blocked GFP–OGT translocation in response to serum (Supplementary Movie 2). Similarly, the addition of wortmannin after stimulation with serum led to rapid dissociation from the plasma membrane (within 3 min), indicating that association with the plasma membrane was dependent on sustained PI(3)K activity (Fig. 2a and Supplementary Movie 3).

We compared the subcellular localization of GFP–OGT with that of a biosensor for PI(3,4,5)P_3, the Akt PH domain fused to a red fluorescent protein (HcRed–Akt_{PH}). Both translocated to and co-localized at the plasma membrane within 30 min after stimulation with serum, indicating their recruitment to the plasma membrane by a common mechanism (Fig. 2b). For both GFP–OGT and HcRed–Akt_{PH}, their associations with the plasma membrane were observed in more than 80% of cells at 5 min after treatment with serum, and the numbers were markedly reduced at 60 min (Fig. 2c). As a control, the localization of GFP was unaffected by serum (Supplementary Fig. 5).

We next examined whether activated PI(3)K is sufficient to drive GFP–OGT redistribution. Expression of a constitutively active PI(3)K led to translocation of GFP–OGT to the plasma membrane even under conditions of serum starvation (Supplementary Movie 4). Again, treatment with wortmannin led to rapid dissociation from the plasma membrane. This result was further supported by our subcellular fractionation experiment, showing that the constitutively active PI(3)K, but not a catalytically dead one, also triggered GFP–OGT accumulation at the plasma membrane (Fig. 2d). As the controls, GFP, the plasma membrane marker Na/K ATPase and the cytoplasmic marker β-actin were not affected (Fig. 2d). Activated PI(3)K is therefore both necessary and sufficient to drive the translocation of GFP–OGT to the plasma membrane.

We examined the subcellular localization of endogenous OGT and Akt. Immunostaining revealed that, similarly to the GFP-tagged proteins, endogenous OGT and Akt were co-localized at the plasma membrane after serum treatment (Supplementary Fig. 6). Consistent with this observation, subcellular fractionation showed that endogenous OGT and Akt accumulated in the plasma membrane fraction in response to insulin but not in the presence of wortmannin (Fig. 2e). The localization of endogenous OGT is therefore regulated by PI(3)K.

Inhibition of PI(3,4,5)P_3 production by PTEN (phosphatase and tensin homolog (mutated in multiple advanced cancers 1)) also markedly decreased the association of OGT with the plasma

membrane (Supplementary Fig. 7), further supporting the notion that $PI(3,4,5)P_3$ recruits OGT to the plasma membrane on insulin signalling.

Finally, we tested whether the K981A/K982A point mutation that abolishes interaction with $PI(3,4,5)P_3$ *in vitro* also affected the response of OGT to $PI(3,4,5)P_3$ *in vivo*. Indeed, the K981A/K982A mutant did not translocate to the plasma membrane in response to stimulation with serum or in response to the expression of the constitutively active PI(3)K (Fig. 2f, and Supplementary Movies 5 and 6). Taken together, these results indicate that OGT is a target of $PI(3,4,5)P_3$ *in vivo*.

O-GlcNAc regulation of insulin signalling

We next sought to dissect events downstream of insulin-stimulated recruitment of OGT to the plasma membrane. In general, the magnitude of *O*-GlcNAc modification of intracellular proteins correlates with extracellular glucose levels[34,35]. We observed that the exposure of 3T3-L1 adipocytes to either high glucose (30 mM) or an *O*-GlcNAcase inhibitor (PUGNAc)—two approaches that increase protein *O*-GlcNAc concentrations—inhibited insulin-stimulated phosphorylation of Akt specifically at Thr 308 but not at another critical phosphorylation site (Ser 473), which is consistent with previous reports[30,40] (Fig. 3a, b). In contrast, phosphorylation of PDK1 at Ser 241 was not affected (Fig. 3a, b). Glucose deprivation had no immediate effect on the phosphorylation of these signalling components (Fig. 3a). We observed that exposing the cells to various concentrations of glucose and insulin did not alter global concentrations of *O*-GlcNAc and phosphotyrosine but rather that of specific proteins (Supplementary Fig. 8).

Concurrent with the decrease in phosphorylation of Akt Thr 308, the increase in intracellular *O*-GlcNAc levels brought about by high concentrations of glucose or by PUGNAc inhibited Akt kinase activity (Fig. 3d). Under the same conditions, extracellular signal-regulated kinase (ERK)1/2 kinase activity remained unchanged (Fig. 3d).

In addition to suppressing Akt phosphorylation and activity, PUGNAc enhanced IRS1 phosphorylation at Ser 307 and Ser 632/635 (Fig. 3b), sites previously shown to mediate the attenuation of insulin signalling[12,13]. In line with these results, adenovirus-mediated overexpression of OGT (Ad-OGT) decreased the phosphorylation of

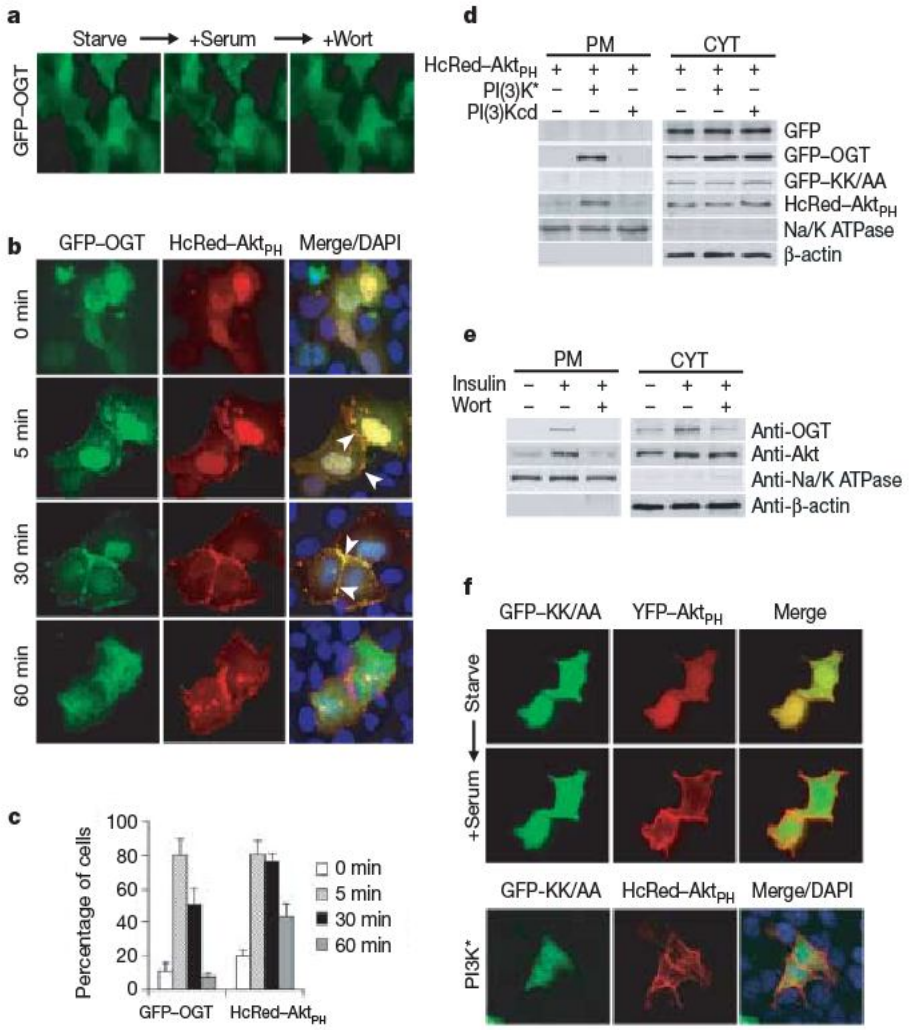

Figure 2 | Phosphoinositide signalling mediates OGT translocation. **a**, Live images of COS-7 cells transfected with the GFP–OGT expression vector, serum-starved overnight and treated with 10% serum and then 100 nM wortmannin (Wort) (from Supplementary Movie 3). **b**, Fluorescent images of fixed COS-7 cells that were co-transfected with the GFP–OGT and HcRed–Akt$_{PH}$ expression vectors followed by treatment with serum for the indicated periods. Right panels, images merged with 4,6-diamidino-2-phenylindole (DAPI) staining of nuclei. Arrows indicate co-localization of GFP–OGT and HcRed–Akt$_{PH}$ on plasma membrane. **c**, Quantification of cells that displayed the plasma membrane localization of GFP–OGT or HcRed–Akt$_{PH}$ after serum treatment for the indicated periods. Error bars show s.e.m. **d**, Immunoblot analysis of subcellular fractionations using anti-GFP, anti-HcRed, anti-Na/K ATPase and anti-β-actin antibodies after COS-7 cells had been transfected with the GFP, GFP–OGT or GFP–KK/AA vector plus the indicated expression vectors. PM, plasma membrane; CYT, cytosol. **e**, Immunoblot analysis of endogenous OGT and Akt in subcellular fractions of 3T3-A14 cells treated with 100 nM insulin minus or plus 100 nM Wort for 30 min. **f**, Top: live images of COS-7 cells co-transfected with the GFP–OGT (KK/AA) mutant and yellow fluorescent protein (YFP)–Akt$_{PH}$, serum-starved overnight and treated with 10% serum (from Supplementary Movie 5). Bottom: images of fixed COS-7 cells co-transfected with the vectors expressing GFP–KK/AA, HcRed–Akt$_{PH}$ and constitutively active PI(3)K (PI(3)K*).

Akt at Thr 308 and its kinase activity, and increased phosphorylation of IRS1 at Ser 307 and Ser 632/635 (Fig. 3c and Supplementary Fig. 9), providing direct evidence for the regulation of the serine/threonine phosphorylation of Akt and IRS1 by *O*-GlcNAc. This regulatory mechanism is not adipocyte-specific, because PUGNAc and Ad-OGT also modulated the phosphorylation of Thr 308 of Akt and Ser 632/635 of IRS1 in Fao hepatoma cells in a PPO-domain-dependent manner (Supplementary Figs 10 and 11). An alteration in *O*-GlcNAc levels had no significant effect on tyrosine phosphorylation of purified IR-β and whole-cell proteins (Fig. 3c, e).

Next we assessed direct targets of OGT in the insulin signalling pathway[41,42]. We found that IR-β and IRS1 were modified by *O*-GlcNAc on stimulation with insulin (Supplementary Fig. 12). Glycosylation of IR-β and IRS1 was highly dynamic, reaching maximum levels at 30 min and declining quickly within 1 h. In contrast, tyrosine phosphorylation of the two proteins was initiated earlier than their glycosylation but was prolonged over 8 h (Fig. 3f). Besides IRS1, other insulin signalling components, including Akt, PDK1 and the p110α subunit of PI(3)K, were detectable in anti-*O*-GlcNAc immunoprecipitates, in which insulin and PUGNAc treatments increased the amounts of glycosylated IRS1 and Akt but not those of PDK1 and p110α (Supplementary Fig. 13). A reciprocal experiment showed that insulin and PUGNAc also increased the glycosylation levels of Akt immunoprecipitates, which is in agreement with a previous report confirming that Akt itself is dynamically modified by *O*-GlcNAc[42] (Supplementary Fig. 14). Taken together, these results indicate that the insulin-induced recruitment of OGT to the plasma membrane imposes *O*-GlcNAc modification on specific signalling components, which in turn influences their phosphorylation level and activity. The half-life of protein phosphorylation in the insulin pathway seems not be affected by *O*-GlcNAc (data not shown).

The ability of *O*-GlcNAc to inhibit Akt phosphorylation and enhance IRS1 serine phosphorylation indicates adverse effects of this modification on insulin signalling. Indeed, increased *O*-GlcNAc modification by PUGNAc inhibited insulin-stimulated glucose transport in 3T3-L1 adipocyte (Supplementary Fig. 15), suggesting that *O*-GlcNAc is involved in attenuation of insulin signalling.

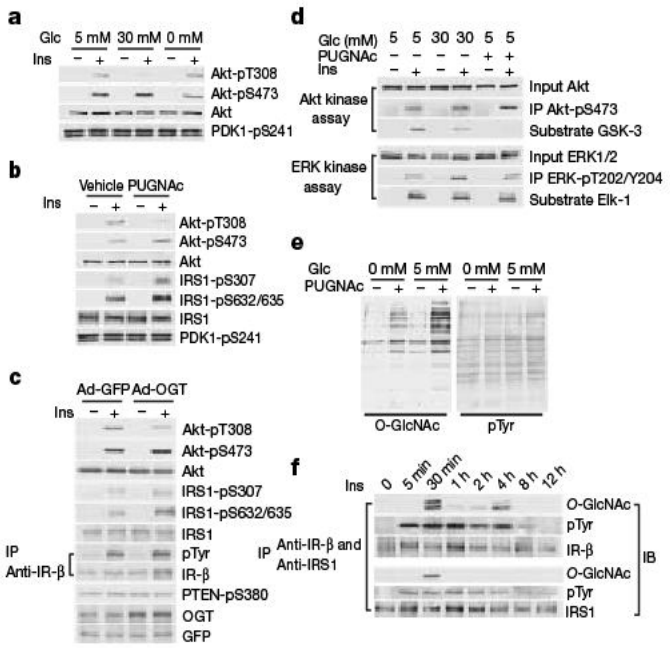

Figure 3 | *O*-GlcNAc dynamically regulates insulin signalling pathway. **a**, **b**, Immunoblot analysis of phosphorylation states and total amounts of the indicated proteins in differentiated 3T3-L1 cells. Cells were incubated for 16 h with various concentrations of glucose (Glc) (**a**) or with 5 mM glucose plus 100 μM PUGNAc (**b**), followed by insulin (Ins) treatment for 30 min. **c**, Immunoblot assay of cell extracts after adenoviral expression of GFP or OGT. Tyrosine phosphorylation (pTyr) was assessed in anti-IR-β immunoprecipitates. IP, immunoprecipitation. **d**, Kinase assays showing the activity of immunoprecipitated phospho-Akt (Ser 473) in phosphorylating the GSK-3α/β crosstide sequence (CGPKGPGRRGRRRTSSFAEG) and the activity of immunoprecipitated phospho-ERK1/2 (Thr 202/Tyr 204) in phosphorylating Elk-1 on the treatments as indicated. **e**, Immunoblot analysis of whole-cell lysates with anti-*O*-GlcNAc antibody (RL2) or anti-phosphotyrosine antibody (4G10) after the indicated treatments for 16 h. **f**, Time course of glycosylation and phosphorylation of IR-β and IRS1. Whole-cell lysates were immunoprecipitated with a mixture of anti-IR-β and anti-IRS1 antibodies, and then immunoblotted (IB) with anti-*O*-GlcNAc antibody (RL2) or anti-phosphotyrosine antibody (4G10).

OGT–lipid interaction mediates insulin resistance

Insulin is a pivotal regulator of carbohydrate and lipid metabolism. To investigate whether OGT modulates insulin signalling in a physiological context, we conducted glucose tolerance tests on C57BL/6J mice with adenoviral delivery of wild-type OGT (Ad-OGT) and the PIP-binding-deficient mutant (Ad-KK/AA) expression constructs to the liver. Glucose excursion curves were similar in Ad-OGT and Ad-KK/AA mice compared with mice transduced with the Ad-GFP control (Fig. 4a). However, Ad-OGT mice had higher levels of plasma insulin and C-peptide than Ad-KK/AA and control mice at 20 min after the glucose challenge (Supplementary Figs 16 and 17), indicating the possible existence of peripheral insulin resistance in Ad-OGT mice with a compensatory increase in insulin release. Indeed, during insulin tolerance tests, Ad-OGT mice showed an impaired decrease in blood glucose at 40 min compared with Ad-KK/AA and control mice (Fig. 4b). Hyperinsulinaemic–euglycaemic glucose clamp experiments showed that the insulin-stimulated glucose disposal rate was similar in Ad-OGT and control mice, indicating that Ad-OGT might not affect the sensitivity of muscle and adipose tissue, the major sites of glucose disposal, to insulin (Fig. 4c). In contract, basal glucose turnover was decreased and the ability of insulin to suppress hepatic glucose production was attenuated in Ad-OGT mice, demonstrating the presence of hepatic insulin resistance (Fig. 4d, e).

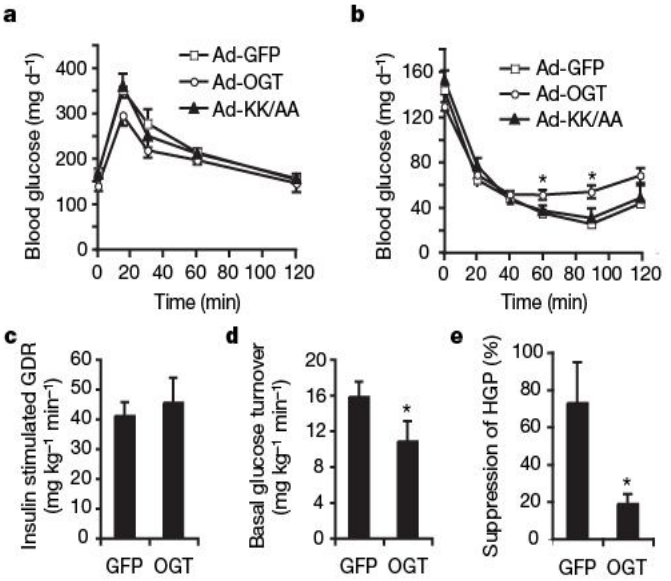

Figure 4 | Hepatic overexpression of OGT produces insulin resistance. **a**, **b**, Glucose (**a**) and insulin (**b**) tolerance tests in 12-week-old C57BL/6J male mice infected with adenovirus expressing GFP or the wild-type or KK/AA mutant of OGT ($n = 6$). Glucose (1.5 g kg^{-1} body weight) (**a**) or insulin (1.5 U kg^{-1} body weight) (**b**) were injected intraperitoneally (i.p.) 6 h after food removal. Asterisk, $P < 0.05$ versus GFP mice. **c**–**e**, Hyperinsulinaemic–euglycaemic glucose clamp studies of 16-week-old mice infected with GFP or OGT adenovirus. Basal glucose turnover ($n = 8$) (**d**), insulin-stimulated glucose disposal rate (GDR) ($n = 7$) (**c**) and percentage suppression of hepatic glucose production (HGP) ($n = 9$) (**e**) were measured. Asterisk, $P < 0.05$ versus GFP mice. Error bars show s.e.m.

Insulin suppresses hepatic glucose production by inhibiting gluconeogenesis and promoting glycolysis and glycogen synthesis. The delivery of Ad-OGT to the liver increased the expression of gluconeogenic genes (those encoding phosphoenolpyruvate carboxykinase and glucose-6-phosphatase) and decreased the expression of glycolytic genes (those encoding glucokinase and glyceraldehyde-3-phosphate dehydrogenase), and these changes were not seen in the mice expressing the KK/AA mutant (Fig. 5a). Moreover, the wild-type Ad-OGT, but not the mutant, decreased glycogen content in the liver, indicating an inhibitory effect of OGT on glycogen synthesis (Fig. 5c).

Insulin promotes lipid synthesis by inducing the expression of lipogenic genes. Ad-OGT expression repressed the level of sterol regulatory element-binding protein-1c (*SREBP-1c*), known as the master regulator of lipogenesis, as well as its target genes including those encoding acetyl-CoA carboxylase 1 (*ACC1*), fatty acid synthase (*FAS*) and stearoyl-CoA desaturase 1 (*SCD1*) (Fig. 5b). This inhibitory effect was abrogated by the KK/AA mutation in OGT (Fig. 5b). The mRNA levels of the genes involved in fatty acid oxidation (those encoding peroxisome-proliferator-activated receptor-α, medium-chain acyl-CoA dehydrogenase and carnitine palmitoyltransferase 1A) and lipid transport (those encoding ATP binding cassette transporters A1, G5 and G8, and apolipoprotein A-I) were unchanged (data not shown).

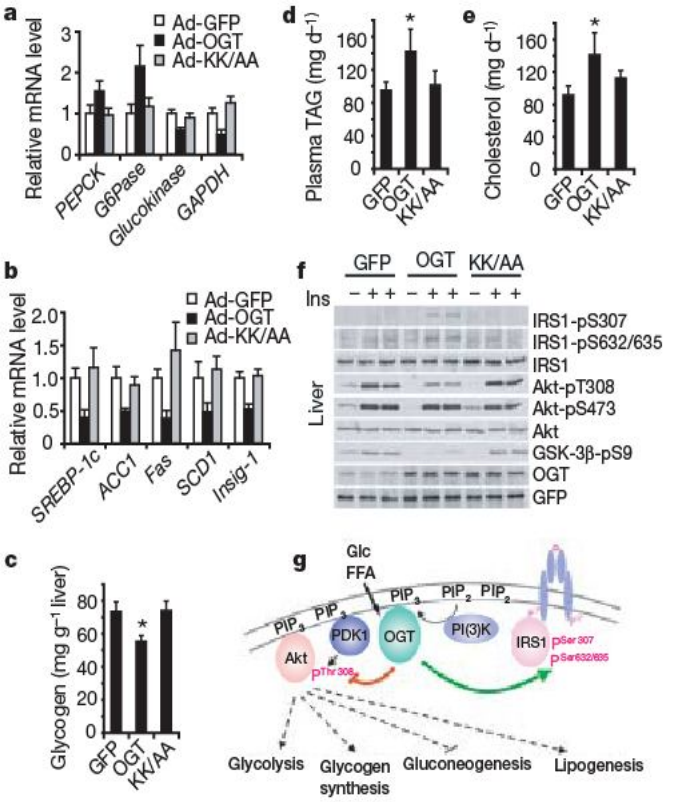

Figure 5 | OGT overexpression causes phosphoinositide-dependent perturbation of insulin signalling. **a**, **b**, Quantitative PCR analysis of gene expression with the use of liver RNA from 6-h-fasted mice infected with the indicated viruses ($n = 4$–6). PEPCK, phosphoenolpyruvate carboxykinase; G6Pase, glucose-6-phosphatase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase. **c**–**e**, Liver glycogen contents, plasma triacylglycerol (TAG) and cholesterol levels in 6-h-fasted mice at 7 days after adenoviral infection ($n = 5$–6). Asterisk, $P < 0.05$ versus GFP mice. Error bars show s.e.m. **f**, Immunoblot analysis of liver extracts from adenovirus-infected mice injected intraperitoneally with insulin (3 U kg^{-1} body weight) or vehicle for 15 min. **g**, Model showing that, under normal physiological conditions, $PI(3,4,5)P_3$ recruits OGT to the plasma membrane in 'phase II', where OGT attenuates insulin signalling by inhibiting phosphorylation at Thr 308 of Akt and promoting IRS1 serine phosphorylation. Excessive quantities of nutrients such as glucose and non-esterified fatty acids aberrantly elevate *O*-GlcNAc levels, thereby impairing insulin action on carbohydrate and lipid metabolism.

Despite the decrease in lipogenic gene expression in the liver, Ad-OGT mice showed increased levels of plasma triacylglycerols and cholesterol in comparison with Ad-GFP and Ad-KK/AA mice (Fig. 5d, e). These results may be associated with repression of the gene encoding Insig-1, a negative regulator of lipid synthesis, by Ad-OGT (Fig. 5b). The concentrations of hepatic triacylglycerol, plasma non-esterified fatty acids, leptin, adiponectin and resistin were similar in the three groups (data not shown).

Our results reveal that hepatic overexpression of OGT impairs the expression of insulin-responsive genes and perturbs glucose and lipid homeostasis in a PPO-domain-dependent manner. Because the KK/AA mutation does not affect the catalytic activity of OGT (Supplementary Fig. 18), these phenotypes are intrinsically dependent on the PIP-binding activity of the enzyme. To ascertain whether this is attributable to defective insulin signal transduction, we examined the phosphorylation state of the insulin pathway. Consistent with our observations in 3T3-L1 and Fao cells (Fig. 3c and Supplementary Fig. 11), Ad-OGT increased insulin-stimulated IRS1 phosphorylation at Ser 307 and Ser 632/635 and decreased Akt phosphorylation at Thr 308 in the liver, whereas Ad-KK/AA had no effect (Fig. 5f). Ser 9 phosphorylation of hepatic glycogen synthase kinase (GSK)-3β was also decreased, which may account for the inhibitory effect of Ad-OGT on glycogen synthesis. In accord with a lack of adenoviral infection in muscle and adipose tissue of Ad-OGT mice, their insulin signalling cascades remained intact (Supplementary Figs 19–21). This leads us to conclude that Ad-OGT induces insulin resistance and dyslipidaemia by PIP-dependent perturbation of insulin signalling.

Discussion

Mounting evidence points to pivotal roles of *O*-GlcNAc modification in regulating diverse functions of nuclear and cytoplasmic proteins[15,37]. Our studies provide the first evidence that this modification mediates critical signalling events at the plasma membrane and identify a previously unrecognized type of $PI(3,4,5)P_3$-binding domain as a critical modulator of this process. Thus, the PPO domain of OGT opens up a target through which phosphoinositide signalling can directly modulate hexosamine signalling and sensitivity.

Activation followed by termination of signal transduction is essential for all signalling pathways. Our studies reveal a new strategy for the attenuation of insulin signal transduction. Insulin stimulates the production of $PI(3,4,5)P_3$ at the plasma membrane, where the lipid recruits PDK1 and Akt to initiate early signalling cascades. As we now show, $PI(3,4,5)P_3$ also recruits OGT to the plasma membrane, where the enzyme acts in a 'phase II' pathway to catalyse dynamic modification of Akt, IRS1 and probably other signalling molecules by *O*-GlcNAc. This inhibits the phosphorylation of Akt at Thr 308 and promotes the phosphorylation of IRS1 at multiple serine residues. As a consequence, OGT modulates the termination (but not the activation) of insulin signalling, hence the term phase II regulation (Fig. 5g). Because *O*-GlcNAc seems not to affect the activities of PI(3)K and PDK1 (X.Y. and R.M.E., unpublished observations), OGT may modulate specific branches in the insulin signalling network.

O-GlcNAc is a putative cellular sensor for systemic metabolic status. Our studies indicate how nutritional cues may regulate insulin signalling through *O*-GlcNAc. Under normal physiological conditions, balanced *O*-GlcNAc levels may confer optimal kinetics of insulin signal transduction. Nutrient excess would lead to aberrant elevation in *O*-GlcNAc levels, which in turn compromise the efficiency of insulin signalling (Fig. 5g). Abnormal *O*-GlcNAc modification of the insulin signalling pathway may therefore contribute to the pathophysiology of insulin resistance, obesity and type 2 diabetes.

METHODS SUMMARY

Protein–lipid overlay assays were performed in accordance with the manufacturer's protocols. Cells were cultured in DMEM medium with 10% FBS except that Fao hepatoma cells were maintained in RPMI 1640 with 10% FBS. 3T3-L1 cell differentiation was induced by an insulin/dexamethasone/isobutylmethyl xanthine cocktail. 3T3-A14 and COS-7 cells were transfected with Transfectin and FuGENE6, respectively. Treatments with various concentrations of glucose and 100 μM PUGNAc were performed in 0.5% BSA for 16 h, followed by stimulation with 10% FBS or 100 nM insulin. Microscopy was performed in fixed or live cells expressing fluorescent fusion proteins. Whole-cell lysates were prepared in RIPA buffer for immunoprecipitation and immunoblotting analyses. C57BL/6J mice were infected with recombinant adenoviruses with the use of systemic injection into the tail vein. Four to six days after viral infection, glucose and insulin tolerance tests were performed in mice that had been fasted for 6 h. After seven days, blood and tissues were collected from 6-h-fasted mice for the measurement of metabolic parameters, quantitative PCR and protein phosphorylation analyses.

Full Methods and any associated references are available in the online version of the paper at www.nature.com/nature.

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Author Contributions X.Y. conceived the project, designed and performed most of the experiments. P.P.O. and S.J.F. participated in protein–lipid binding and cell imaging experiments. P.D.M. performed hyperinsulinaemic–euglycaemic glucose clamp studies. J.C.H. assisted in biochemical and animal experiments. F.Z. performed OGT activity assays. W.V.S. performed bioinformatic analyses. J.M.O., R.H.M., J.E.K. and S.J.F. provided intellectual input and technical expertise. R.M.E. supervised the project. X.Y. and R.M.E. wrote the manuscript.

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LETTERS

Energetic neutral atoms as the explanation for the high-velocity hydrogen around HD 209458b

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Absorption in the stellar Lyman-α (Lyα) line observed during the transit of the extrasolar planet HD 209458b in front of its host star reveals high-velocity atomic hydrogen at great distances from the planet[1,2]. This has been interpreted as hydrogen atoms escaping from the planet's exosphere[1,3], possibly undergoing hydrodynamic blow-off[4], and being accelerated by stellar radiation pressure. Energetic neutral atoms around Solar System planets have been observed to form from charge exchange between solar wind protons and neutral hydrogen from the planetary exospheres[5–7], however, and this process also should occur around extrasolar planets. Here we show that the measured transit-associated Lyα absorption can be explained by the interaction between the exosphere of HD 209458b and the stellar wind, and that radiation pressure alone cannot explain the observations. As the stellar wind protons are the source of the observed energetic neutral atoms, this provides a way of probing stellar wind conditions, and our model suggests a slow and hot stellar wind near HD 209458b at the time of the observations.

Energetic neutral atoms (ENAs) are produced wherever energetic ions meet a neutral atmosphere, and solar wind ENAs have been observed at every planet in the Solar System where ENA instrumentation has been available—for Earth[5], Mars[6] and Venus[7].

By energetic we mean that the ions have a much greater velocity than the thermal velocities of the exospheric neutrals. During the charge exchange process, an electron is transferred from the neutral to the ion, resulting in a neutral atom and an ionized neutral. Because of the large relative velocities of the ions and the exospheric neutrals, the momenta of the individual atoms are preserved to a good approximation. Thus, the produced ENAs will have the same velocity distribution as the source population of ions.

When first observed (also by their Lyα signature[8,9]), the extended hydrogen coronae of Mars and Venus were assumed to constitute the uppermost layers of an escaping exosphere. The observed densities were used to infer exospheric scale heights and temperatures, which proved to be extremely high compared with theoretical predictions (up to 700 K). *In situ* spacecraft observations[10] later found exospheric temperatures of ~210 and ~270 K. The discrepancy was eventually explained by photochemically produced energetic particles, and by ENAs, produced by charge exchange between energetic solar wind protons and the planetary exosphere. Although this mechanism is well known in the Solar System, it has not been considered as a possible origin of the atomic hydrogen corona revealed by Hubble Space Telescope observations of HD 209458b.

HD 209458b is a Jupiter-type gas giant with a mass of $\sim 0.65 M_{\rm Jup}$ and a size of $\sim 1.32 R_{\rm Jup}$ that orbits at ~0.045 AU (ref. 11) around its host star HD 209458, which is a solar-like G-type star with an age of about 4 Gyr. The activity of the star can be estimated from its X-ray luminosity measured by the XMM-Newton space observatory, and is comparable to that of the present Sun during a moderately quiet phase[12]. Because of its Sun-like stellar type and average activity, it is reasonable to use the energy environment observed at the Sun as inputs for our model.

For a first estimate of the ENA production near HD 209458b, we assume that the charge exchange takes place in an undisturbed stellar wind that is flowing radially away from the star. At 0.045 AU from HD 209458, the stellar wind is most likely subsonic[13] and does not produce a planetary bow shock. Simulations indicate a subsolar magnetopause distance of about four planetary radii if the planet is magnetized[14]. If the planet is not magnetized, we would expect the undisturbed stellar wind to get even closer to the planet. Here we model the ENA production by a particle model that includes stellar wind protons and atomic hydrogen. Charge exchange between stellar wind protons and exospheric hydrogen atoms takes place outside a conic obstacle that represents the magnetosphere of the planet (Supplementary Fig. 1). The resulting exospheric cloud, along with the produced ENAs, covers a region larger than the stellar disk, as seen from Earth (Fig. 1). The cloud is shaped like a comet tail owing to the stellar radiation pressure, curved by the Coriolis force, as predicted[15] and seen in earlier numerical simulations[1,3]. There is a population of atoms with high velocity—these are the stellar wind protons that have charge-exchanged, becoming ENAs. In the velocity spectrum along the x axis (the planet–star line), the ENAs are clearly visible as a distribution that is separate from the main exospheric hydrogen component, because of the different bulk velocities and temperatures (Fig. 2).

Now we estimate how the ENA cloud would affect the observed Lyα absorption spectrum of HD 209458b[1]. The line profile was observed outside and during transit, and the difference between the two profiles corresponds to the attenuation by hydrogen atoms (Fig. 3).

There are several features of the transit spectrum that any proposed source of the observed hydrogen atoms needs to account for. First, there are hydrogen atoms with velocities of up to $-130\,{\rm km\,s^{-1}}$ (away from the star). Second, there is a fairly uniform absorption over the whole velocity range -130 to $-45\,{\rm km\,s^{-1}}$. Third, there is absorption in the velocity range between 30 and $105\,{\rm km\,s^{-1}}$ (towards the star).

The current explanation of the observation is that hydrogen atoms in the exosphere are undergoing hydrodynamic escape, and are further accelerated by the stellar radiation pressure[1,4]. But there are difficulties in explaining the observations by this process, as can be seen by examining the three features listed above.

First, a large radiation pressure on the hydrogen atoms is needed to accelerate them to a velocity of $130\,{\rm km\,s^{-1}}$ before they are

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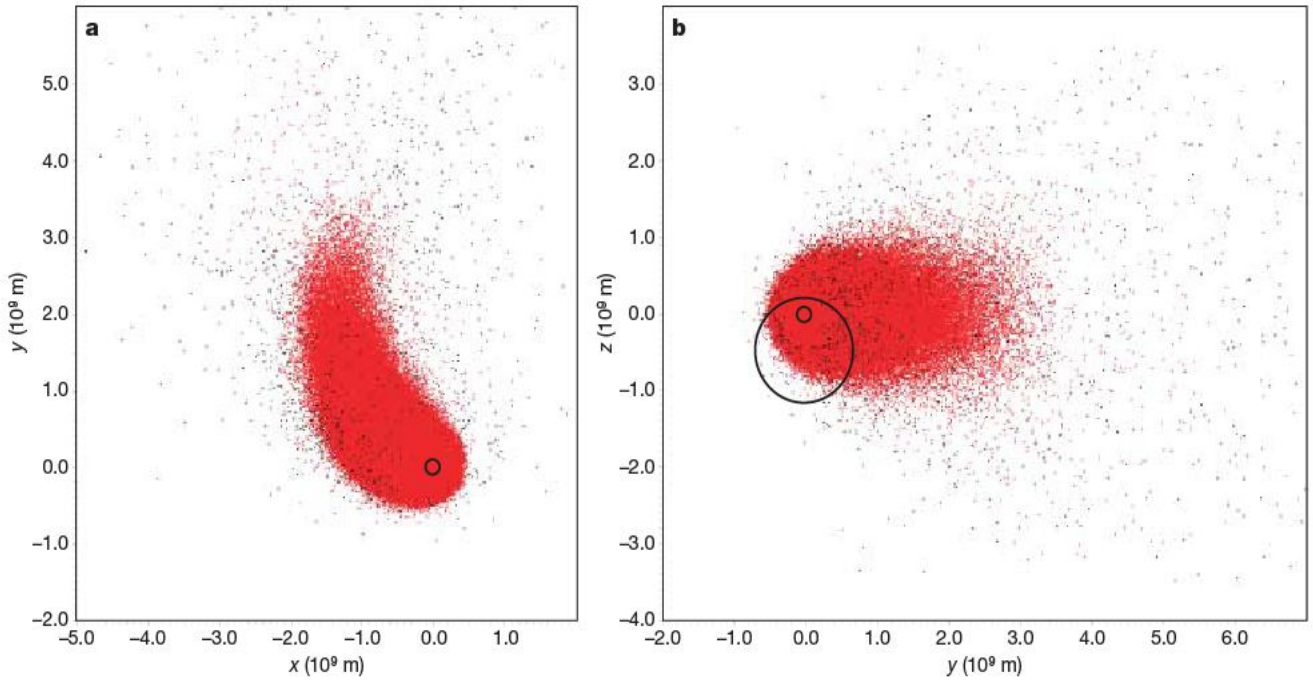

Figure 1 | The hydrogen cloud around the planet. **a**, Shown from above, perpendicular to the planet's orbital plane; **b**, as seen from Earth, along the direction of the x axis. Each point corresponds to a hydrogen meta particle. The colour of the points shows the velocity of the particles along the x axis. Particles with velocity magnitude smaller than 50 km s^{-1} are red, and those with higher velocity are black. The small circles show the planet size. The large circle in **b** shows the star's position at mid-transit. During transit the star moves from left to right in **b**. At the outer boundaries of the simulation domain, stellar wind protons are injected with a number density of 2×10^3 cm^{-3}, velocity 50 km s^{-1} and temperature 10^6 K. The planet's interaction with the stellar wind is modelled by removing all stellar wind protons inside a conic obstacle at a substellar distance of about $4.2R_p$ (where the radius of the planet $R_p = 9.4 \times 10^7$ m). Hydrogen atoms are launched from an inner boundary (a sphere of radius $2.1R_p$) assuming a number density of 10^8 cm^{-3} and a temperature of 7,000 K, consistent with atmospheric models[18]. The trajectory of each proton and hydrogen atom is followed in time. The forces on a hydrogen atom are the gravity of the planet, the Coriolis force due to the rotating coordinate system, and radiation pressure. After each time step a hydrogen atom can undergo photoionization, elastic collision with another hydrogen atom or charge exchange with a proton. The photoionization time assumed is 4 h which is a scaled Earth value. The radiation pressure corresponds to a photon–hydrogen collision rate of 0.35 s^{-1} and is chosen to improve the model fit. It is lower than a scaled Earth value of 0.6–1.6 s^{-1} over a solar cycle. The coordinate system used is centred at the planet with its x axis towards the star, and the y axis opposite to the planet's velocity. Further details of the simulations can be found in the Supplementary Information.

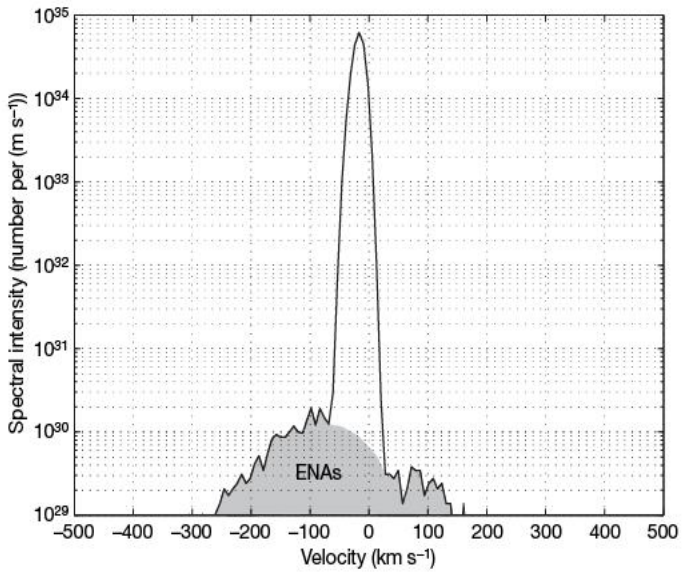

Figure 2 | Velocities of the hydrogen atoms. The modelled x axis (planet–star) velocity spectrum of hydrogen atoms in front of the star at the moment of mid-transit, not including atoms in front or behind the planet. The part of the distribution that is due to ENAs is shaded. Varying the stellar wind temperature and velocity in the model confirms that the width of this part of the distribution is proportional to the temperature of the stellar wind, with a larger width for larger temperatures, and the centre of the distribution follows the stellar wind velocity. The unshaded part of the spectrum is due to the exospheric hydrogen atoms.

photoionized. The acceleration must occur before they move out from the region in front of the star, owing to the orbital motion of the planet. The second feature is difficult to explain. If hydrogen atoms were driven to speeds of up to 130 km s^{-1}, we would expect the velocity spectrum to have an exponential decay for higher velocities, because photoionization gives the hydrogen atoms a finite lifetime (four hours on average). This drop-off for high velocities is independent of the details of the model, for example the values of radiation pressure and photoionization lifetime used. This would lead to a decay in the absorption spectrum, inconsistent with the observed fairly uniform absorption over the whole velocity range -130 to -45 km s^{-1}. Finally, an exosphere driven by radiation pressure cannot explain hydrogen atoms moving towards the star with speeds between 30 and 105 km s^{-1}. However, this feature is not completely certain, and more observations may be needed to clarify whether an absorption is present in the red part of the line (towards the star)[1].

Our model shows that the three observed features can be explained by ENAs. If we turn off the ENA production in the model, none of these features are explained. When we compare the modelled Lyα profile with the observed ones, we find that the modelled spectrum leads to attenuation over the whole velocity range from -130 to -45 km s^{-1}, as observed. The model also shows some absorption in the red part of the velocity spectrum, that is, hydrogen atoms moving at high velocities towards the star, because for this stellar wind (50 km s^{-1} and 10^6 K), some part of the proton velocity distribution will have positive velocities along the x axis, resulting in an ENA flux towards the star. This slow and hot stellar wind is not unrealistic at such small orbital distances[13].

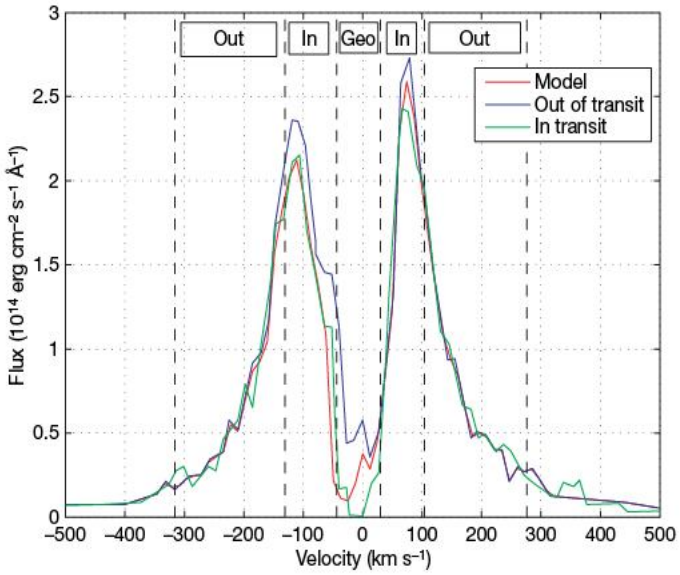

Figure 3 | Comparison of modelled with observed Lyα profiles. Blue, the observed profile before transit. Green, the observed profile during transit. Red, the modelled profile, constructed by applying the attenuations computed from the simulations to the observed profile before transit. The abscissa is the hydrogen velocity along the x axis (away from Earth, towards the star). The regions where there is a significant difference between the profiles are denoted 'In' and 'Geo', the latter being the region of geocoronal emission at low velocities that should be excluded. The modelled profile is computed at the instant of mid-transit. The details of computing the Lyα attenuation from the hydrogen cloud are given in the Supplementary Information. The modelled Lyα absorption shown here is for a stellar wind velocity of 50 km s^{-1} and a temperature of 10^6 K. The fit is worse for stellar wind velocities of 0 or 100 km s^{-1}, or stellar wind temperatures of 2×10^6 K or 0.5×10^6 K (see Supplementary Information).

If ENAs from charge exchange are responsible for the observed attenuation, we have, on the one hand, much less information on the main exospheric component than suggested by previous explanations. Because what we observe are mainly ENAs, a range of exospheric conditions and atmospheric loss rates can be consistent with the observation. The observation only constrains atmospheric escape insofar as the exosphere has to be extended enough to reach the stellar wind outside the planet's magnetopause. The atmospheric escape through ENA production is small. For the model parameters used here, the loss is 7×10^5 kg s^{-1}, more than an order of magnitude smaller than the estimated thermal loss of about 10^7 kg s^{-1} for similar exospheric conditions[16–18].

On the other hand, we gain information on the underlying plasma flows, and if this is the undisturbed stellar wind, we have a way of observing stellar wind properties such as temperature and velocity around other stars, at the location of extrasolar planets. By varying the stellar wind temperature, the stellar wind velocity and the radiation pressure in the model, we find a best fit of the modelled Lyα absorption to the observation for a stellar wind velocity of 50 km s^{-1}, and a temperature of 10^6 K (Fig. 3).

Although, for HD 209458b, the available Lyα data are affected by large uncertainties, more accurate observations would improve the derived stellar wind estimates. Also, the depletion of the stellar wind proton flow by charge exchange will change the character of the interaction between planet and stellar wind. Present models of the stellar wind interaction with HD 209458b have not taken this process into account[14]. More observations of the Lyα absorption by HD 209458b, and its variation over time, could be used to confirm the origin of the extended hot hydrogen cloud. There should be more variability on short timescales for absorption by ENAs than for other explanations, such as hydrodynamic escape, because stellar wind parameters can change significantly on a timescale of hours, as seen near Earth.

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Author Contributions A.E. wrote an initial version of the simulation code. F.S. helped in modelling the observation. T.P. provided knowledge on the atmospheres of extrasolar planets. H.L. suggested that the HST observation could be due to ENAs. P.W. contributed expertise in ENA processes

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LETTERS

Martian stepped-delta formation by rapid water release

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Deltas and alluvial fans preserved on the surface of Mars provide an important record of surface water flow[1–3]. Understanding how surface water flow could have produced the observed morphology is fundamental to understanding the history of water on Mars. To date, morphological studies have provided only minimum time estimates for the longevity of martian hydrologic events, which range from decades to millions of years[4–7]. Here we use sand flume studies to show that the distinct morphology of martian stepped (terraced) deltas[8–11] could only have originated from a single basin-filling event on a timescale of tens of years. Stepped deltas therefore provide a minimum and maximum constraint on the duration and magnitude of some surface flows on Mars. We estimate that the amount of water required to fill the basin and deposit the delta is comparable to the amount of water discharged by large terrestrial rivers, such as the Mississippi. The massive discharge, short timescale, and the associated short canyon lengths favour the hypothesis that stepped fans are terraced delta deposits draped over an alluvial fan and formed by water released suddenly from subsurface storage.

Some martian fans have a distinctive 'stair step' topography[7–11] (Fig. 1) that might have formed by volcanic flows[10], erosive wave action[8,11], repeated alluvial fan deposition[9], or mass wasting[1]. In a permeable sediment flume (Fig. 2), large, short-duration flows build fans draped with stepped delta deposits in a mock crater filling with water. We use observed martian morphology combined with physical modelling to constrain the maximum and minimum duration of the martian hydrologic events on the basis of sediment transport. To scale sediment mobility, we use the carrying capacity of the water flow (discharge and depth[12]) to compare across the large spatial and temporal differences between Mars and a flume; maintaining similar Shields particle mobility number and a Froude number close to unity[13] ensures a similarity of process during the formation of the large-scale morphological features.

The experimental stepped deltas are produced in 24 min in three separate stages that depend on rising water level and on fluctuating discharge of sediment and water, despite constant experimental conditions (Fig. 2). Initial erosion of the steep crater lip by small, high-concentration, debris-like flows formed a cone-shaped alluvial fan (Fig. 2d). As canyon slope decreased, subaerial sheet-flows[14] buried the initial fan under a broader fan that contained the bulk of the sediment volume (Fig. 2e). Sedimentation changed to deltaic deposition only when a decrease in infiltration resulted in a rise of the water table above the crater floor (Fig. 2c, at 8 min). As the basin filled, infiltration significantly decreased and water discharge became steady, even though sediment discharge continued to fluctuate because of bank erosion during canyon widening and incision. Thus, distinctive topographic steps formed at the intersection of the shoreline and the fan apron, similar to other experiments[15].

These experiments and two other aspects of the martian fan morphology tightly constrain the formation of stepped topography. First, the delta apex is not incised, so the feeder canyon must have ceased

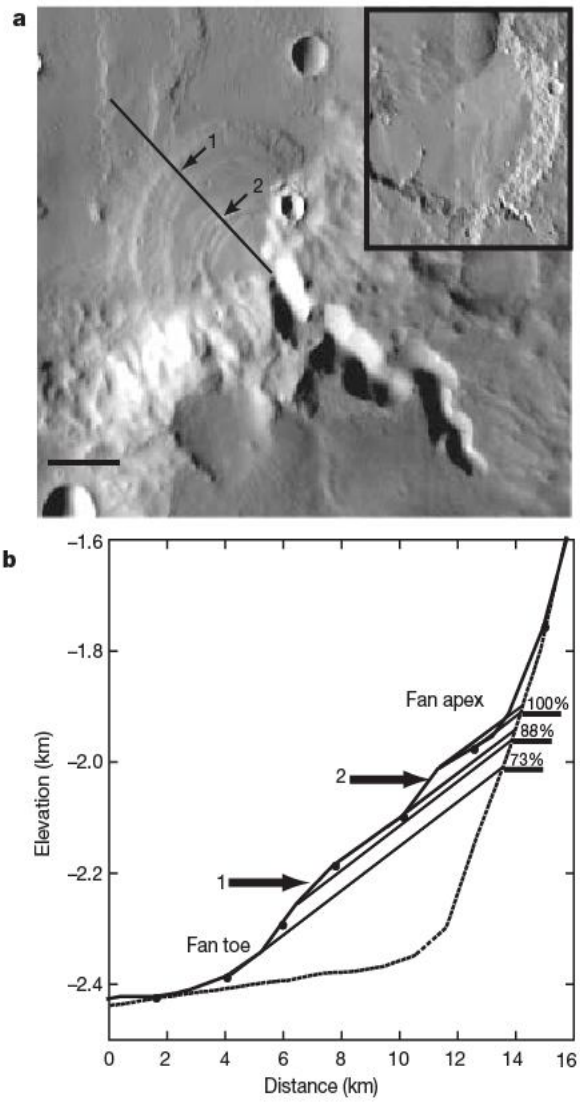

Figure 1 | Example of a stepped delta in a martian crater at 8° S, 200° E. **a**, Mosaic of Thermal Emission Imaging System (THEMIS) images (resolution 18 m per pixel). Scale bar, 2.5 km. Arrows indicate the two most distinct steps of the terraces; solid line shows the transect modelled in **b**. First-order channel is 20 km with no developed drainage network. Inset, regional setting. **b**, Topographic results. Solid line, slope of delta; dashed line, average rim slope (from three profiles). Arrows indicate steps 1 and 2. The volume of the fan through time (as 73%, 88% and 100%) is estimated from the alluvial surface covered by the lobe deposits.

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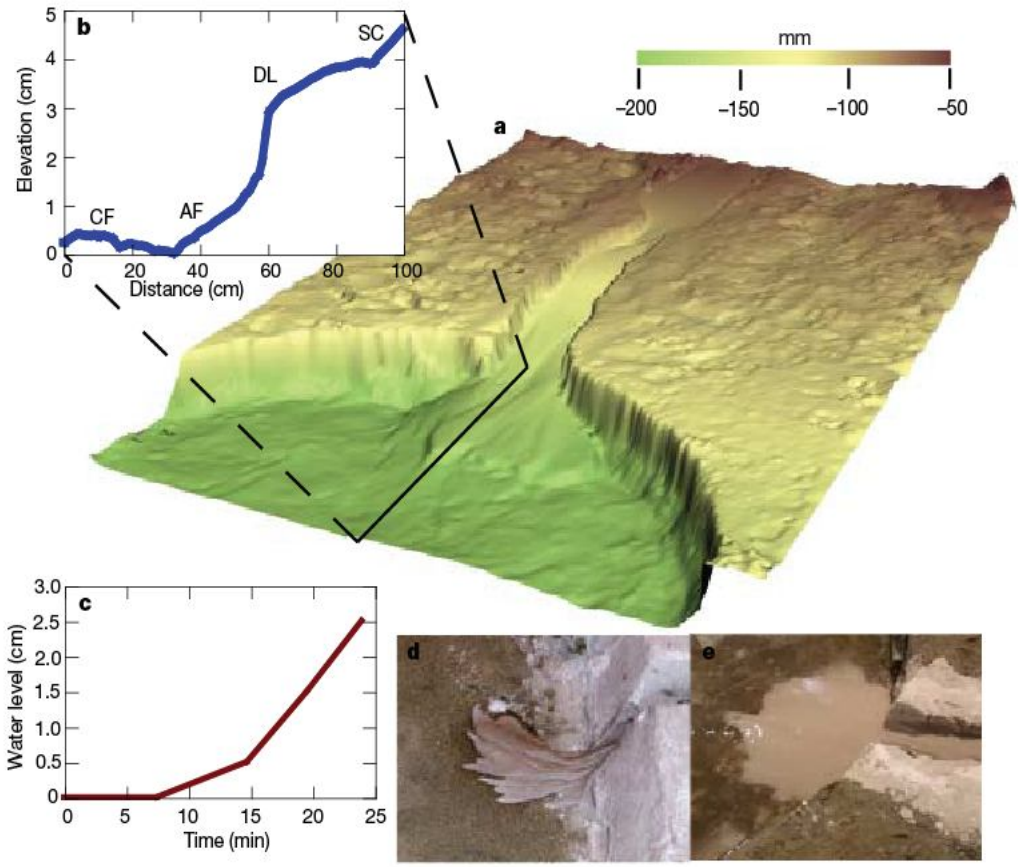

Figure 2 | Experimental delta deposited into a crater. The colour-coded digital terrain model (**a**; colour bar shows mm below measuring surface) and fan profile (**b**) clearly show the transition from the crater floor (CF) into an alluvial fan (AF) with a prominent delta lobe (DL) transitioning to the feeding stream canyon (SC). High infiltration rates caused a slow rise in water level followed by an abrupt increase following local saturation (**c**). Initially, sediment was transported in concentrated flows (**d**) then, as stream power decreased, the fan transitioned to sheet flow deposition (**e**).

supplying water before the basin began draining. Second, sedimentation must have ceased rapidly to prevent long-term deposition from prograding over the stepped topography. Thus the unique driving conditions (single event, no long-term deposition), combined with morphological analysis and sediment transport modelling, permit calculation of the formation time for the martian fan (Fig. 1), which is the ratio of water (or sediment) volume to the transport rate.

Using standard water discharge and bedload equations[16], we estimate the martian and experimental sediment and water timescales by calculating the time to deposit the fan sediment or fill the basin with water. In the experiment, water discharge predicts a formation time of 12 min; sediment discharge predicts a formation time of 118 min. The experimental formation time was 24 min; therefore, the discharge estimate is 50% less than the actual time—a reflection of unaccounted infiltration losses. The bedload transport estimate overpredicts the timescale by 5 times—a reflection of the importance of *en masse* sediment transport observed early in the experiment. Indeed, in sheet flows on steep, terrestrial fans, it is observed that much of the coarse sediment is suspended in dense, *en masse*-moving slurries[17,18], which decrease the net transport time significantly.

We estimate the minimum amount of water required to fill the martian crater at 6.4×10^3 km^3, based on an assumed radius of 64 km given erosion of the northern rim. We use minimum and maximum estimates for channel width, depth and slope, because discharge equations are most sensitive to channel dimensions, which are uncertain for Mars. For the maximum flow estimate (minimum time), we assume the current canyon is nearly full (1,200 m wide by 60 m deep) and the current canyon slope (0.044). The maximum assumed water discharge is 8.1×10^5 m^3 s^{-1}, 5 times the discharge of the Amazon River (1.5×10^5 m^3 s^{-1})[19]. Assuming this high, constant flow was responsible for filling the basin and depositing the delta, the formation time ranges from 0.2 yr from water discharge and 1.0 yr from sediment discharge (Table 1). For the minimum flow estimate (maximum time), we assume a smaller active channel in the canyon; we take values in agreement with gravel-transporting rivers on Earth (for example, the Wairoa and Rakaia rivers in New Zealand[19]) and estimate the channel dimensions to be 100 m wide by 5 m deep. The consequent water discharge of 2.2×10^3 m^3 s^{-1} corresponds to a delta formation timescale of ~90 yr, as estimated from both water and sediment volume (Table 1). Smaller water discharges (up to the point that transport halts) have correspondingly longer timescales, and cause an unrealistic mismatch between water and sediment transport rates.

We can develop a depositional history for the delta body (Table 1, Figs 1b, 3) because the steps record distinct moments when the shoreline and sediment transport intersect and the surfaces of the experimental stepped terraces have the same slope as the original, underlying alluvial fan (Fig. 2b). The volume of the delta segments (Fig. 1b) lies above the relict depositional surface produced by projecting the alluvial fan slope headward (Fig. 1b). In the martian example, we use the average slope deduced from the entire population of large martian alluvial fans (0.04)[2] because the surface area is too small for accurate slope measurements from spacecraft

Table 1 | Estimated feature formation times

	Feature formation time (yr)	
	Maximum	Minimum
Sediment: deposit fan	90	1.0
Sediment: erode canyon	130	1.4
Water: fill basin	93	0.20
Alluvial fan: sediment	66	0.74
Alluvial fan: water	18	0.04
Step 1: sediment	14	0.16
Step 1: water	39	0.08
Step 2: sediment	10	0.11
Step 2: water	54	0.11

Data are for stepped fan sections.

data. In the experimental case, we use direct measurements of the exposed alluvial surface. We assume fan thickness to be approximately uniform along any cross-section from the apex to the toe. Given the challenges of calculating the three-dimensional volume of an alluvial fan[20] and the uncertainty of the pre-existing topography (in the martian case), the fan is approximated in only two dimensions. In both the martian and the experimental cases, most of the sediment was deposited before the intersection of the water level and fan apron. In the martian case, 73% of the sediment volume of the stepped delta body was deposited before step formation while the remainder was deposited in lobes as the shoreline prograded up the apron (Figs 1b, 3). This parallels the experimental deltas where the small sediment volume deposited during terrace formation dominates the morphology (Figs 2, 3).

For the martian maximum discharge estimate, the time required to deposit the alluvial fan segment (0.74 yr) is 19 times longer than the time required to fill the basin with water to that elevation (0.04 yr). This agrees with our experimental observations—the assumption of bedload transport in the sediment discharge calculation is not appropriate early in the depositional history of the stepped delta when high-concentration mass flows are likely. However, bedload transport is a good assumption during step deposition where the sediment discharge timescale (0.1 yr, step 2) agrees with the water discharge timescale (0.1 yr, step 2). (Steps 1 and 2 are shown in Fig.1.) Assuming the low-discharge (maximum time) case, the sediment discharge timescale for alluvial fan formation (66 yr) is also higher than the water discharge timescale of formation (18 yr) but only by 4 times (Table 1). Whereas the timescales for the formation of the total delta by bedload transport begin to merge at lower water discharge values, the timescales for deposition of the terraces become unrealistically long: for instance, for step 2, the estimate is ~19 yr (sediment) and ~53 yr (water). This implies that there would be some 30 yr of sediment starved flow where the water would entrain bedload causing incision into the sediments, which is not observed.

One could argue that the discharge had been shut down and restarted after some time. But leakage through the crater floor would then cause a fall of the water level and subsequent incision, channelization and related delta lobe formation[21]. Although shallow incisions may not be visible in current imagery, the much larger size of protruding, 'telescoping' delta lobes that would form at the outlet of incised channels would be easily detected on the available imagery. Any undetected and, thus, relatively small incisions would not significantly change the formation time we calculate here. Similarly, healing of former larger channel incisions during a fall-rise cycle is, in theory, possible, but should also be detectable on the basis of the delta lobes on the terraces. In a completely sealed basin with no atmospheric loss, it would be possible to cease water flow, pause sediment deposition, and restart flow without changing the stepped morphology as we describe above. Determining the rate of water loss is virtually impossible; permeability of the martian soil is unknown but is probably very high, especially in highly fractured impact basins[22,23]. Evaporation rates vary from 33 m yr^{-1} to 0.16 m yr^{-1}, depending on temperature, air pressure and wind speed[24,25], all of which are unknown for the time the delta formed. We know experimentally that the stepped morphology requires that the water level never dropped or stabilized for a significant period of time. Therefore, we assume a 50% discharge loss rate as an upper limit; this results in a total water loss per metre squared of 2,000–30 m yr^{-1} to evaporation and infiltration combined. Under these conditions, the maximum formation time doubles but the ratios of sediment to water discharge remain reasonable, maintaining realistic sediment transport conditions and, therefore, the stepped morphology.

Stepped deltas formed during a single hydrologic event of short duration, and provide unique insight into a two-sided constraint on water discharge and formation time. The estimated water volumes and timescales indicate water discharges on a par with some of the largest terrestrial fluvial systems. For example, to fill the entire crater basin in Fig. 1 (6,500 km^3 of water) requires a decadal timescale with a discharge between that of the Rhine River (70 km^3 per decade; 93 yr filling timescale) and the Mississippi River (5,000 km^3 per decade; 13 yr filling timescale)[19]. These discharge estimates must be reconciled with a martian canyon length hundreds of times smaller than comparable terrestrial rivers, as well as a lack of a developed drainage network. Thus, the stepped morphology and our modelling of a large, rapid, single event argue against surface precipitation and favour a hypothesis of release from subsurface water storage. These results must be integrated with other sedimentary bodies on Mars (especially fans that appear to have formed from surface precipitation) as well as with the interior processes required to store and release large quantities of water.

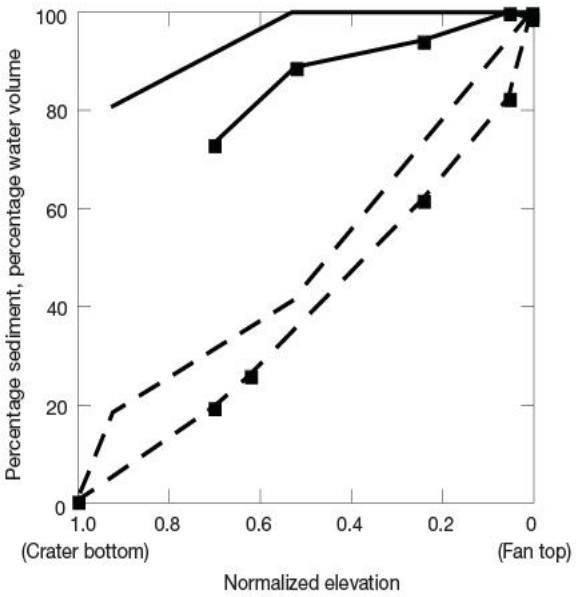

Figure 3 | The depositional history for experimental and martian stepped fans. The normalized elevation of the delta in the crater is plotted against the percentage of water (solid lines) and sediment (dashed lines) for the martian example (filled squares; Fig. 1) and the experimental (no symbols) fans. In both cases, the majority of the sediment was deposited before the intersection of the water and the fan apron, and the sediment volume contained in the lobes is minimal compared to that of the alluvial fan.

METHODS SUMMARY

The experiments were performed in the Eurotank, a large sand flume, at Utrecht University. Digital terrain models (DTMs) produced through photogrammetry of the surface have a vertical accuracy of better than 250 μm (horizontal) and 100 μm (vertical). We also took video (Supplementary Movie) and still photography. A mock crater measuring 2.1 m × 1.6 m × 0.1 m was constructed on a 2.7 m × 5 m sand-covered surface sloping 0.03 (Methods). An initial shallow and small relief channel (1.5 m × ~0.02 m) routed water flow into the crater at the start of the experiment. Water (400 l h^{-1}) and sediment (2.5 l h^{-1}) were constantly discharged into the feeder channel. However, the natural processes of infiltration, canyon widening, and incision drove fan formation. The initial regional water table (within the flume) was just below the bottom of the crater floor. Initially, water infiltrated into both channel walls and crater floor, saturating the sediment and raising the local water table (Supplementary Movie). The sediment yield at the outlet of the canyon experienced large fluctuations because of bank erosion and channel forming processes. The Supplementary Movie shows channel migration, bank erosion, and upstream knickpoint migration, all of which contributed to large pulses in sediment discharge. Following the experimental runs, the regional water table was dropped and the water drained by seepage without disturbing the morphology.

Full Methods and any associated references are available in the online version of the paper at www.nature.com/nature.

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Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

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Author Contributions Experimental design was developed and sediment transport analysis conducted by E.R.K., M.v.D. and G.P. Experimental runs and data analysis were conducted by E.R.K. and M.v.D. Timescale analysis was conducted by E.R.K. and M.G.K. E.R.K. wrote the paper. All authors discussed the results and commented on the manuscript.

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LETTERS

Self-healing and thermoreversible rubber from supramolecular assembly

Philippe Cordier[1], François Tournilhac[1], Corinne Soulié-Ziakovic[1] & Ludwik Leibler[1]

Rubbers exhibit enormous extensibility up to several hundred per cent, compared with a few per cent for ordinary solids, and have the ability to recover their original shape and dimensions on release of stress[1,2]. Rubber elasticity is a property of macromolecules that are either covalently cross-linked[1,2] or connected in a network by physical associations such as small glassy or crystalline domains[3–5], ionic aggregates[6] or multiple hydrogen bonds[7–16]. Covalent cross-links or strong physical associations prevent flow and creep. Here we design and synthesize molecules that associate together to form both chains and cross-links via hydrogen bonds. The system shows recoverable extensibility up to several hundred per cent and little creep under load. In striking contrast to conventional cross-linked or thermoreversible rubbers made of macromolecules, these systems, when broken or cut, can be simply repaired by bringing together fractured surfaces to self-heal at room temperature. Repaired samples recuperate their enormous extensibility. The process of breaking and healing can be repeated many times. These materials can be easily processed, re-used and recycled. Their unique self-repairing properties, the simplicity of their synthesis, their availability from renewable resources and the low cost of raw ingredients (fatty acids and urea) bode well for future applications.

An attractive method of designing small-molecule systems exhibiting rubber-like elasticity is to use the concept of supramolecular polymers[17]. Indeed, ditopic molecules—molecules able to directionally associate with two other molecules—can form long-lived chains and show polymer-like behaviour both in solutions[9] and in bulk[18–20] (see also ref. 21 and references therein). By mixing ditopic and multitopic molecules—which are able to associate with more than two other molecules—a network could be formed[10,17] (Fig. 1). Thus far, in bulk, designed small molecules that can form such supramolecular networks flow or partially crystallize and behave like plastic resins or fibres (ref. 21 and references therein, and ref. 22). Rubber-like behaviour has not been reported.

To avoid crystallization while keeping strong directional interaction is a challenge in the route towards supramolecular rubbers from small molecules. We propose the use of mixtures of molecules bearing a variety of strongly associating groups. We rely on the variety of molecular architectures to render crystallization difficult and on both entropy of mixing and directional specific interactions to avoid the macroscopic phase separation of different species. For this purpose, as a starting material we use fatty dimer acids made from natural renewable resources (vegetable oils) available in bulk quantities and various compositions[23]. They are liquid at room temperature and in contrast to other classical diacids they do not crystallize, forming glasses instead. The glass transition can be varied by dosing the oil from which fatty diacids are made, and even more interestingly, these starting materials can contain variable amounts of trimer acids. We have thus solved the problem of the synthetic availability of multitopic molecules. Indeed, our strategy is to use carboxylic-acid ends to attach functional groups able to form multiple hydrogen bonds. Our two-step synthetic pathway (Fig. 2) introduces three types of functional groups able to strongly associate via multiple hydrogen bonds, namely amidoethyl imidazolidone, di(amido ethyl) urea and diamido tetraethyl triurea.

In the first step, acid groups were condensed with a controlled excess of diethylene triamine. In the second step, the obtained product was reacted with urea. The resulting compound, called **A**, resembles a translucent glassy plastic. When heated to 90 °C, well above its glass transition temperature ($T_g = 28$ °C), it behaves like a soft rubber with strain at breaking of about 350% and it completely recovers its dimensions after being deformed to 100% strain (Supplementary Fig. 1). At still higher temperatures, above 160 °C, the material flows like a viscoelastic liquid and can be moulded, extruded and (re)shaped. Compound **A** is soluble in benzyl alcohol and

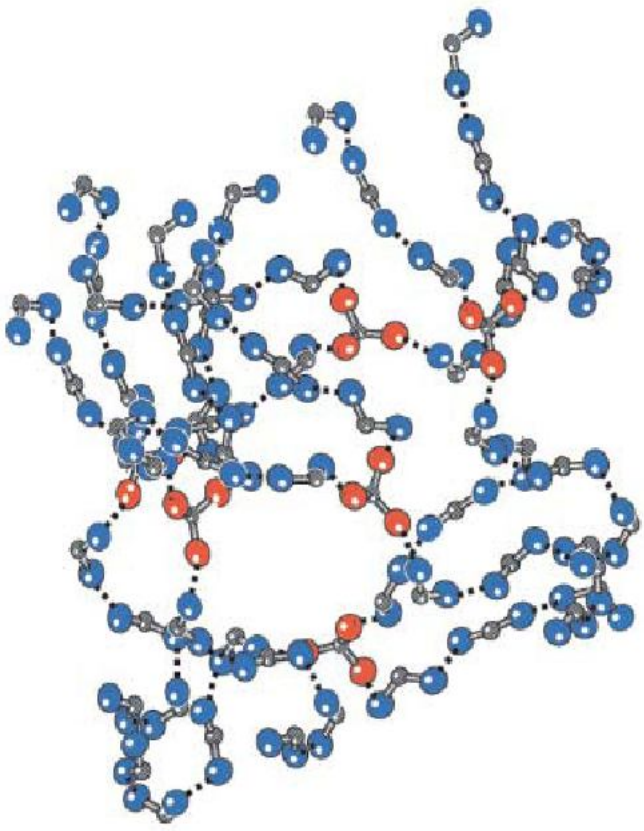

Figure 1 | Supramolecular network. Schematic view of a reversible network formed by mixtures of ditopic (blue) and tritopic (red) molecules associating by directional interactions (represented by dotted lines).

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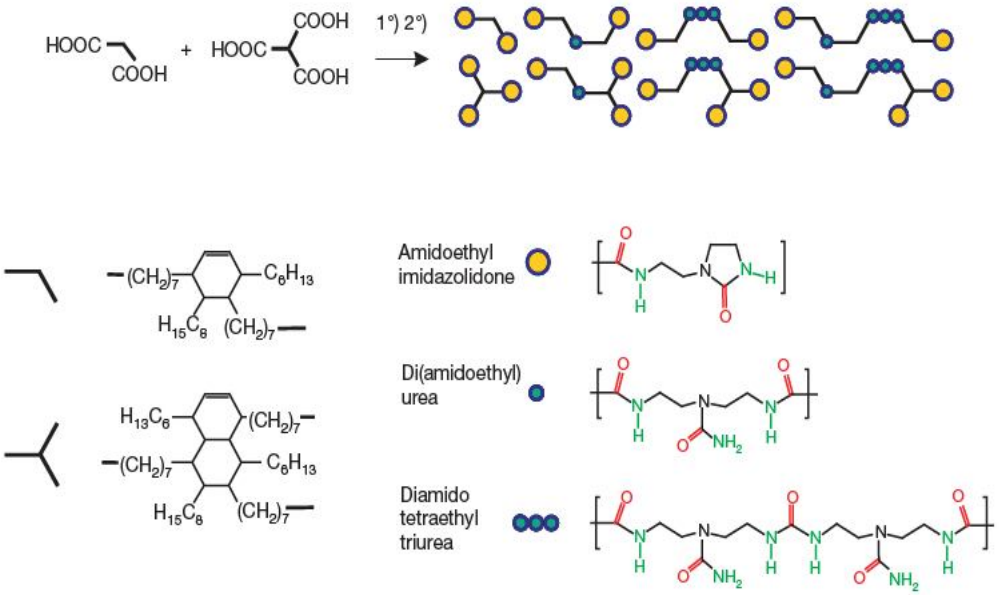

Figure 2 | Synthesis pathway. A mixture of fatty diacid and triacid is condensed first with diethylene triamine and then reacted with urea giving a mixture of oligomers equipped with complementary hydrogen bonding groups: amidoethyl imidazolidone, di(amidoethyl) urea and diamido tetraethyl triurea. The hydrogen bond acceptors are shown in red, donors in green.

methanol/chloroform mixtures and insoluble in water, with water uptake of ~14 weight %. The narrowness and detectable fine structure of nuclear magnetic resonance (NMR) signals (Supplementary Fig. 2) reveal that A is made of small molecules: oligomers. This is confirmed by size exclusion chromatography and intrinsic viscosity measurements (Supplementary Fig. 3). Infrared spectroscopy shows the presence of multiple hydrogen bonds between N–H and C=O groups (Supplementary Fig. 4).

To lower the glass transition temperature, we plasticized compound A with varying quantities of dodecane. With 11% w/w dodecane, T_g is about 8 °C and the resulting compound, called **B**, is a non-tacky rubber-like material. Differential scanning calorimetry and X-ray scattering confirm that the material is not crystalline (Supplementary Fig. 5). A broad scattering event at 0.17 $Å^{-1}$ indicates a short-range organization with characteristic length of 36 Å, comparable with the molecular size. The frequency dependence of the oscillatory shear moduli for storage, G', and loss, G'', clearly shows the polymer-like behaviour (Fig. 3a). Its behaviour at high frequencies indicates the proximity of the glass transition, whereas at low frequencies, the elastic rubbery character dominates. At plateau, the storage shear modulus is about 3×10^4 Pa and the relaxation time extrapolated from these data are not less than 3×10^6 s (a few weeks) at 50 °C.

The rubber-like properties of this material are confirmed at large deformations. Figure 3b shows stress–strain curves that resemble those of soft rubbers. The strain at break exceeds 500%. After elongation to 300% with a speed of 2.5 mm min^{-1} and release of stress, the residual strain is less than 5%. When the cycle is repeated, the sample recovers completely without any residual strain. Finally, the inset of Fig. 3b shows that during uniaxial deformation the volume of the material is preserved just as for ordinary rubbers.

Figure 3c shows the results of creep experiments performed at 50 °C. When a stress of 5×10^3 Pa is applied for 8×10^4 s the strain is about 32% and it increases at a rate of 0.04% per hour. This value is consistent with the relaxation time extrapolated from linear rheology. When the applied stress is released, the sample completely recovers its dimensions; the residual strain is negligible, less than 5×10^{-2}%. For a higher load of 2×10^4 Pa applied for as long as

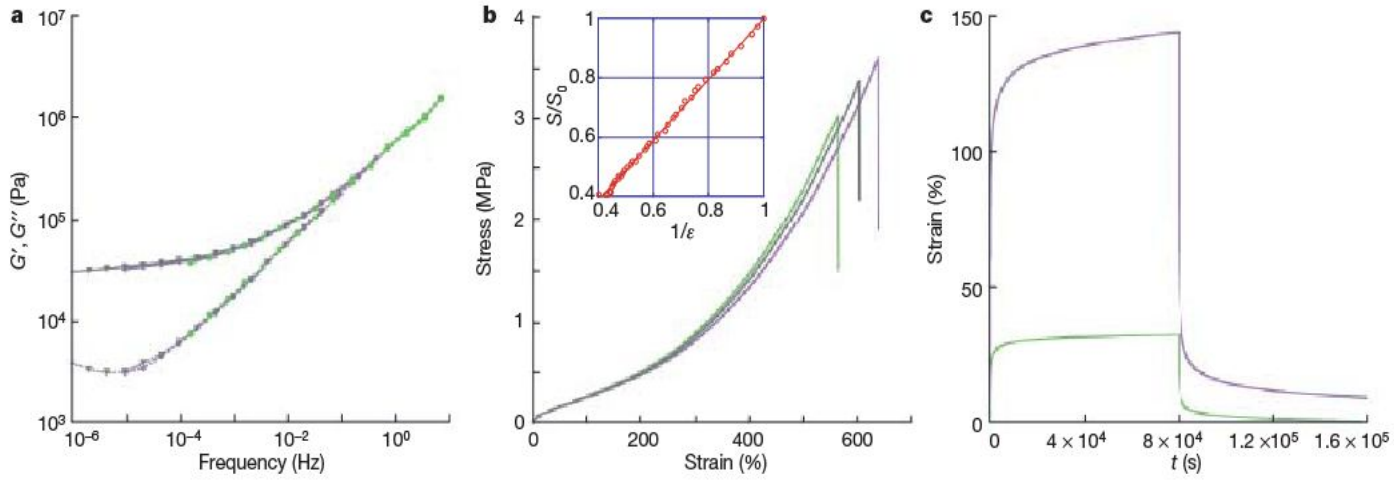

Figure 3 | Rheological and mechanical properties. **a**, Frequency dependence of the storage (top, G') and loss (bottom, G'') moduli of compound **B** obtained by classical time–temperature superposition shifts. The reference temperature is 50 °C and measurements were performed at 50 °C (green circles), 70 °C (purple diamonds) and 90 °C (black triangles). **b**, Stress–strain curve of supramolecular rubber **B**. Data for three samples are shown to illustrate the reproducibility. The inset shows that the cross-section area varies as the inverse of the tensile deformation. During deformation the sample volume is conserved as in conventional rubbers. **c**, Creep–recovery experiment of compound **B** for an applied stress of 5,000 Pa (green) and 20,000 Pa (purple).

8×10^4 s, the strain is about 144% and it increases by 0.36% per hour. After release of the stress, the residual strain is less than 8% (but even after 8×10^4 s, the relaxation still continues). Tensile stress relaxation experiments (Supplementary Fig. 6) confirm this picture. Because of the proximity of the glass transition, viscous dissipations are important in both stress and strain relaxation experiments and samples recover their dimensions rather slowly.

All the above mechanical features show that material **B**—although it is made of oligomers—behaves very much like a rubber. In contrast to classical rubbers, however, material **B** exhibits unique self-healing properties: when a sample is broken or cut into pieces and the pieces are brought into contact together for some time at room temperature (20 °C) they self-heal without the need to heat or press strongly. The mended samples are able to sustain large deformations and recover their shape and size when stress is released. Figure 4a illustrates that longer healing times lead to better healing, but even when contact time is as short as fifteen minutes a repaired sample can be deformed up to about 200% without breaking. Interestingly, for all healing times, stress–strain curves superpose and only show elongation at break changes. Although healed scars are not visible, repaired samples break at the scar location except after long healing times (Supplementary movie 1). The cycle of stretching, breaking and healing can be repeated many times.

These observations seem to confirm our design principles: to be self-mending the supramolecular rubber has to be made from small molecules and the supramolecular associations have to be strong and long-lived so that at equilibrium, the fraction of non-associated groups in the network is low. However, the strength of the associations has to be lower than that of covalent bonds so that when broken, many non-associated groups are present near the fracture surface. Self-healing is efficient because a large number of groups 'eager' to link is available. At shorter healing times fewer bridges across the interface are formed and the elongation at break is lower.

Of course, the above mending mechanism also implies that when the sample is not mended immediately after being broken but only after some waiting time, the number of non-associated groups available for healing decreases. Indeed, during the waiting time, some free groups find partners within the broken part. But, as shown in Fig. 4b and c, broken samples can still efficiently heal after waiting for 6 h or even 18 h. When the waiting time is varied, the stress–strain curves again superpose and the elongation at break of mended samples remains impressive.

There is a maximum waiting time after which self-healing is no longer possible in a reasonable time. During this maximum waiting time thermal equilibrium is reached, and so few free groups remain available for partner exchange across the scar and for bridging. To confirm this mechanism, we increased the temperature at which samples were held during waiting time and then performed the healing operation after cooling the pieces back to room temperature. The maximum waiting time after which healing is no longer possible decreased, from more than one week at 23 °C, to about 48 h for waiting time at 40 °C, 95 min at 60 °C, 15 min at 90 °C, and 5 min at 120 °C. This is because equilibrium is reached much more quickly when temperature is raised, and so broken associating groups find partners within the broken piece much faster. Finally, to confirm that it is necessary to have non-equilibrated free groups on both of the surfaces to be joined together, we checked that a freshly cut surface does not adhere to the surface of a non-broken sample at room temperature.

It is difficult to obtain direct spectroscopic evidence of an excess of non-associated hydrogen bonds at a freshly cut surface because even reflectance infrared techniques probe and average over a depth comparable to the radiation wavelength, that is, a few micrometres. Nevertheless, using time-dependent infrared spectroscopy we were able to confirm the slow dynamics of hydrogen-bond re-association (which we consider to be the mechanism of self-healing). This

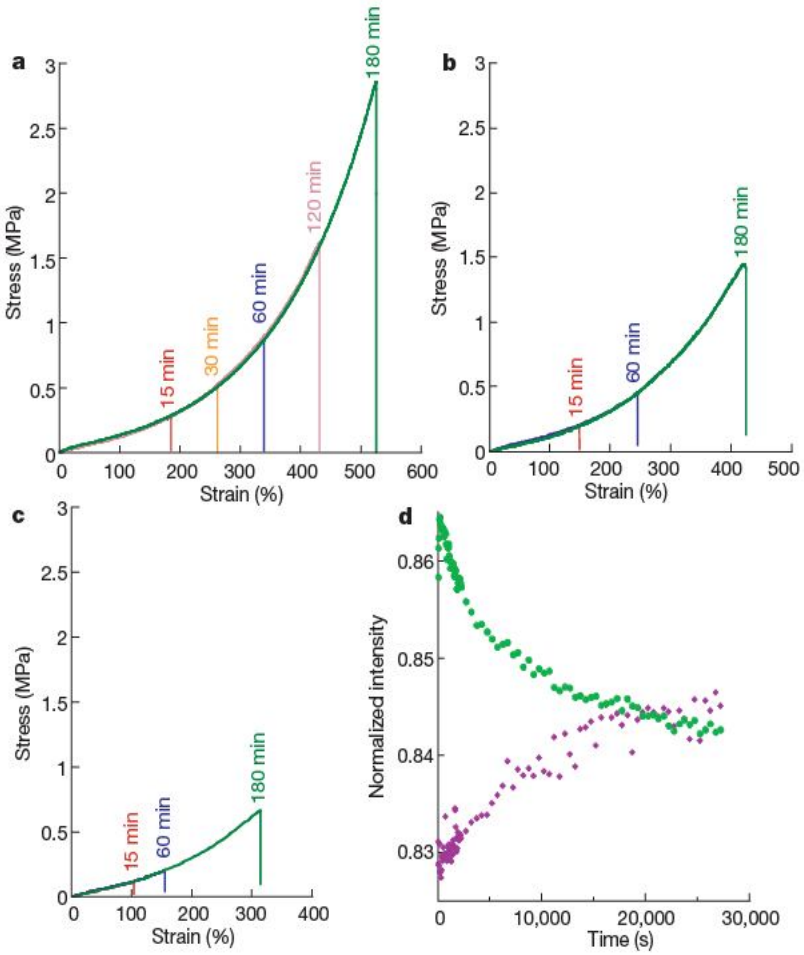

Figure 4 | Self-mending at room temperature. **a**, Cut parts are brought into contact at 20 °C immediately after being cut (waiting time less than 5 min). Curves represent stress–strain behaviour measured for convenience at 40 °C after different healing times. **b**, Stress–strain behaviour at 40 °C of mended samples; mending was performed at 20 °C after keeping broken samples apart for 6 h. **c**, As in **b** but cut samples were kept apart for 18 h and then mended at 20 °C. Coloured vertical lines in **a** to **c** correspond to elongation at breaking for given healing times (for all healing times, stress–strain curves superpose almost exactly and show elongation only at break changes). **d**, Time-dependent infrared experiments. The sample was heated to 125 °C for 10 min and then quenched to 25 °C. Infrared absorption spectra evolutions were recorded. The intensity at 1,524 cm^{-1}, characteristic of free N–H bending motions (green) decreases, whereas the intensity at 1,561 cm^{-1}, characteristic of associated N–H bending motions (purple) increases. These data confirm the long lifetime of open hydrogen bonds when they are created in excess.

slowness accounts for the most striking feature of self-healing materials: their ability to mend rapidly even after a rather long waiting time, even though the samples do not flow (creep) at these timescales.

Indeed, in a temperature-jump experiment (Fig. 4d), the sample was heated at 125 °C for 10 min and then quickly cooled down to 25 °C and the evolution of the infrared spectrum was observed. Figure 4d shows that characteristic bands of free N–H and bound N–H bending motions evolve in opposite directions with a long characteristic time of about 10^4 s. A similar evolution was observed for C=O stretching bands, thus confirming that when excess free hydrogen groups are created, they associate slowly and the association times are compatible with times during which healing is possible. We emphasize that the characteristic decay time of the number of non-associated bonds is much smaller than the mechanical relaxation time. The ratio of these two times is expected to be high for strongly associating systems, especially when some short-range organization is present[24].

Compared to covalently crosslinked rubbers, the supramolecular rubbers described here cannot indefinitely hold stress without creep (even though characteristic relaxation times can be as long as weeks) and the strain recovery is slow. However, different compromises can be achieved while retaining unique self-mending and high extensibility characteristics. For example, we found that when water rather than dodecane is used as plasticizer the glass transition is lowered to −15 °C and 500% strain is completely recovered in a few seconds when stress is released, much as in conventional rubbers, and stretched samples can maintain stress for about ten hours without appreciable creep (Supplementary movie 2). It is also possible to attach other associating groups to fatty di- and tri-acids, instead of the three groups proposed here, and to vary the energy and lifetime of associations. Finally, the diversity of fatty acids made from vegetable oils and the additional flexibility of choosing the diacid/triacid ratio provides a useful tool with which to tune properties. This versatility is not only an asset for industrial development but also offers the opportunity to improve our understanding of the challenging physics of unusual elasticity, glass-like dynamics and relaxations, adhesion and supramolecular structures formation[25–30].

METHODS SUMMARY

Compound **A** is obtained at the 100 g scale in two steps. 175 g of Empol 1016 supplied by Cognis (mixture of 4% monoacid, 79% diacid, 17% triacid and polyacids) was condensed with 70.3 g of diethylenetriamine at 160 °C under nitrogen over 24 h. 72 g of *oligo*-amidoamine with a [$\underline{CH_2}$-CONH] to [$\underline{CH_2}$-NH_2] ratio of 1.8 as determined by NMR obtained after elimination of unreacted amine (chloroform/water extractions) was then reacted with 17 g of urea at 135–160 °C for 7.5 h under nitrogen, whereupon ammonia and unreacted urea were extracted by vacuum stripping and water washings. The material was dried under vacuum and pressed at 120 °C into 100 cm^2 area × 2 mm thickness steel moulds. Swelling with dodecane was achieved at 60 °C over 24 h.

^1H, ^{13}C and correlation NMR spectra were recorded from $CDCl_3/CD_3OH$ mixtures (50/50 by volume) using a 400 MHz Bruker spectrometer. Infrared spectra were recorded using a Bruker Tensor 37 Fourier transform-infrared spectrometer (4 cm^{-1} resolution) equipped with a Specac Golden Gate ATR heating cell. Thermal analyses were performed at a heating rate of 10 °C per min under helium with a TA Instruments DSC Q1000 apparatus. Size exclusion chromatography experiments were performed at 40 °C in $CHCl_3$/BzOH at a flow rate of 1 ml per min using a Waters set-up equipped with a differential viscometer and a differential refractometer.

ARES Rheometrics was used for rheological frequency sweeps (10^{-2} to 10 Hz) with 1% applied strain in the parallel plates geometry. Creep–recovery tests were performed using a Haake RS100 rheometer. Tensile tests were performed on ISO 527-3 normalized specimens using an Instron 5564 apparatus. The Poisson ratio was determined by measuring the sample cross-section and deformation at fixed time intervals using a digital camera. Healing experiments were performed at room temperature (20 °C) by bringing cut samples together and pressing for less than 15 s. The pressure applied by hands was about 6×10^4 Pa.

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Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

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LETTERS

Evidence of lower-mantle slab penetration phases in plate motions

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It is well accepted that subduction of the cold lithosphere is a crucial component of the Earth's plate tectonic style of mantle convection. But whether and how subducting plates penetrate into the lower mantle is the subject of continuing debate, which has substantial implications for the chemical and thermal evolution of the mantle[1,2]. Here we identify lower-mantle slab penetration events by comparing Cenozoic plate motions at the Earth's main subduction zones[3] with motions predicted by fully dynamic models of the upper-mantle phase of subduction, driven solely by downgoing plate density[4]. Whereas subduction of older, intrinsically denser, lithosphere occurs at rates consistent with the model, younger lithosphere (of ages less than about 60 Myr) often subducts up to two times faster, while trench motions are very low. We conclude that the most likely explanation is that older lithosphere, subducting under significant trench retreat, tends to lie down flat above the transition to the high-viscosity lower mantle, whereas younger lithosphere, which is less able to drive trench retreat and deforms more readily, buckles and thickens. Slab thickening enhances buoyancy (volume times density) and thereby Stokes sinking velocity, thus facilitating fast lower-mantle penetration. Such an interpretation is consistent with seismic images of the distribution of subducted material in upper and lower mantle[5,6]. Thus we identify a direct expression of time-dependent flow between the upper and lower mantle.

Seismic methods image anomalies at locations of present and past subduction in upper and lower mantle. These correlate quite well with the history of subduction extending back to 200 Myr ago, if lower-mantle slab sinking rates average about 1–2 cm yr^{-1} (refs 7, 8). Only in a few locations do slab anomalies continue directly into the lower mantle, and mostly they do not extend to depths much larger than about 1,500 km (refs 5, 6, 9, 10). Elsewhere, slabs flatten and deform near the base of the upper mantle[5,6,11,12], and lower-mantle anomalies are unconnected. Seismic methods can only image the current state of the mantle, and do not reveal when material entered the lower mantle, or even whether the lower-mantle anomalies are the result of thermal coupling rather than mass flux[13].

The one to two orders of magnitude increase in viscosity and endothermic phase change that comprise the transition from upper to lower mantle both hamper flow into the lower mantle. Numerical models have shown that this can lead to time-dependent lower-mantle slab penetration[14–18], where subduction occurs in several stages. At first, subduction under rapid trench retreat results in pooling of material at the base of the upper mantle[19]. Then, when a sufficiently large mass has accumulated, it sinks into the lower mantle, accompanied by a strong increase in subduction velocity and drop in trench retreat rates[17,18].

No one has previously looked for such signatures in past plate motions, and, even for the present day, it is debated what governs the motions[20,21]. It is generally agreed that subduction is driven by downgoing-plate negative buoyancy. Yet most present-day subduction velocities and slab dips display little correlation with plate age, the main control on buoyancy, and other forces have been proposed to play an important part[3,20,21]. Also, it is not understood why most of today's subduction velocities are confined to a range of about 4–9 cm yr^{-1}, in spite of a large variation in plate sizes, and regional tectonics[20].

Using a fully dynamic model (see Supplementary Information and ref. 4), we have characterized plate motions and morphologies during the first subduction phase (when it is confined to the upper mantle) for a freely subducting plate. Free subduction is the most basic form of subduction, driven solely by downgoing plate buoyancy and resisted passively by the mantle and an overriding plate. These models provide a baseline to distinguish phases of upper-mantle-confined subduction from phases where subduction may be penetrating the lower mantle. Most other subduction models are not fully dynamic and impose Earth-like plate or trench motions. But to understand what controls these motions, fully dynamic models—where plate motions and morphology can adjust self-consistently—are required.

The main free subduction characteristics emerging from our models[4] are: (1) slab sinking velocities, v_{sink}, are Stokes velocities, that is, they are controlled only by slab density and shape, and by mantle viscosity[4,14]. For realistic effective plate viscosities, two to three orders of magnitude stronger than the upper mantle (see, for example, refs 20, 22), forces that would try to push plates down faster than this will result in slab deformation and thickening, thereby increasing slab Stokes velocity, while forces that try to hold the slab back (for example, resistance to plate bending at the trench), will result in slab detachment or viscous dripping. (2) The plate's strength generally does not allow it to bend to a vertical dip on the timescale it takes to sink into the mantle. As a result, plates with a higher strength, but the same slab buoyancy (that is, same sinking velocity), subduct at a smaller dip, and faster rate, v_{sub}. (3) Free subduction occurs basically by trench retreat, at a rate[4,23] $v_{tr} = v_{sub}\cos\alpha_d = v_{sink}\tan\alpha_d$, where the dip α_d is that of the downgoing slab between 100 and 400 km depth, that is, below the bend. Thus, like v_{sub}, trench retreat increases with plate density and with plate strength. (4) Plate advance, v_{ad}, only occurs to the extent that it is required to preserve continuity between the unsubducted and subducted plate, that is, for a plate that does not stretch or thicken, $v_{ad} = v_{sub} - v_{tr} = v_{sink}[1 - \cos\alpha_d]/\sin\alpha_d$. Thus, the highest possible rate of free plate advance (at $\alpha_d = 90°$, and $v_{tr} = 0$) is the Stokes sinking velocity. Factors that increase the ratio of plate advance over trench retreat are ridge push (the gravitational sliding of plates from the ridge to the trench), a low-viscosity asthenosphere at the base of the plate, and large plate widths[4,24–26]. The few other published dynamic free subduction models display subduction styles similar to ours, where

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conditions are the same[17,18,23,25–27]. Where other models, or natural subduction zones, display behaviour different from these free subduction characteristics, external forces/constraints play a part.

We compare our model velocities with the Cenozoic motions at the world's main subduction zones from the recent update of the global lithospheric age–plate velocity data base of ref. 3. Natural subduction is characterized by low trench retreat, with rates averaging 0.1–0.3 times plate advance rates, throughout the Cenozoic. Furthermore, trench motions are often episodic, and display expressions of outside forcing[21] (Supplementary Information). This explains why a plate-buoyancy signature has only been recognized in present-day absolute plate advance rates (that is, motions of the downgoing plate in a hotspot-reference frame)[28]. Recent data[3,21] confirm the age trend, and it is compatible with an upper-mantle Stokes sinking velocity limit on plate advance, like our models predict for free subduction (Fig. 1). The limiting Stokes velocities for a very reasonable average upper-mantle viscosity of $(0.5–1.0) \times 10^{21}$ Pa s bracket most of today's slab sinking velocities, as estimated from surface motions and deep dips (Supplementary Fig. 2). An age trend is less apparent in convergence velocities, which are the sum of plate-advance and trench motions, because of the variability of the latter (Supplementary Information).

The range of present-day subduction and plate-advance motions is limited, as in our models (where subduction is driven only by upper-mantle slab buoyancy), with one clear exception. Under Central America, sinking and plate-advance velocities are 40–100% higher than expected for the young age of the plate at the Middle America trench, which should give it a buoyancy barely negative enough for self-sustained subduction (Fig. 1, Supplementary Information). These high velocities require an enhanced Stokes velocity, implying either a very low-viscosity upper mantle (about an order of magnitude lower than everywhere else, which seems unreasonable), or increased mass as in the case of lower-mantle penetration. This last explanation is compatible with seismic tomography, where the anomaly attributed to subduction under Central America is the only relatively unambiguous example of a continuous slab-like anomaly into the mid to deep lower mantle[6,10].

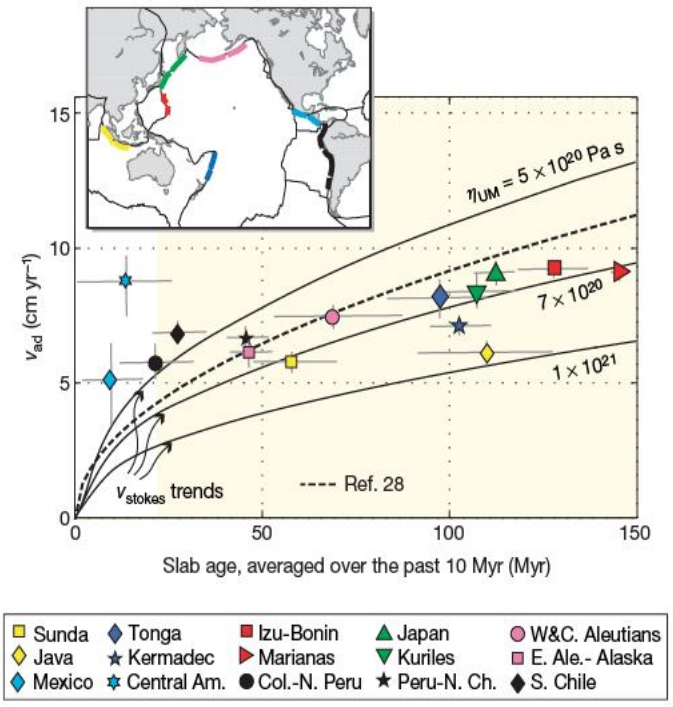

Figure 1 | Present-day plate-advance velocities. Main panel, absolute downgoing plate motions, v_{ad}, versus upper-mantle slab age (plate age at the trench averaged over the last 10 Myr of subduction) from the compilation of ref. 3 compared with Stokes velocities for upper-mantle slabs (1,000 km width and upper-mantle viscosity, η_{UM}, of $(1.0, 0.7, 0.5) \times 10^{21}$ Pa s (or constant η_{UM} of 1.0×10^{21} Pa s and plate widths of 1,000, 2,000, 3,000 km, respectively). For comparison with the data, model densities were converted to equivalent plate ages using the parameters from ref. 30. For slab ages less than 20 Myr, subduction is probably not self-sustaining (white background). The error bars represent the full range of variation within each trench segment. Trends are similar when slab age is averaged over 5 or 15 Myr. The trend of ref. 28 also fits the recent data presented here. Inset, map of the zone segments used. Segmentation scale corresponds to scale at which the main variations in plate motions and dip occur. Figure key gives names. Abbreviations: Ale., Aleutians; Am., America; Ch., Chile; Col., Columbia; N., North; S., South; E., East; W&C., West and Central.

The Cenozoic history of motions at the major Pacific and Southeast Asian subduction zones[3] contains more examples of very high plate-advance rates (dips and therefore sinking velocities are not known back in time), all associated with subduction of relatively young lithosphere. And not only the advance velocities, which carry uncertainties associated with the choice of hotspot-reference frame, but also the (reference-frame independent) subduction velocities strongly exceed model rates at these times (Supplementary Fig. 4). The clearest examples of upper-mantle slab-buoyancy driven and faster modes of subduction are shown in Fig. 2.

The Japan-Kurile-Kamchatka and Aleutians-Alaska zones (designated Japan and Alaska hereafter) (Fig. 2a and b) display a clear switch from one mode to another. In both zones, advance velocities for the past 25 Myr lie within the Stokes velocities for upper-mantle confined slabs that fit present-day sinking velocities (Fig. 1). Japan's recent velocities cluster along a single Stokes trend, while Alaska's define a somewhat higher and less tightly clustered age trend. Before 25 Myr ago, there is no age trend and velocities are 1.5–2 times higher than those of similarly aged lithosphere subducted along these trenches today, and 2–3 times higher than the model trend through the recent Japan velocities. Subduction velocities below Middle America have been excessively high for its age throughout most of the past 55 Myr (Fig. 2c). Only between 50 and 55 Myr ago, and for the northern part of the Cocos subduction in the past 10 Myr, do the values approach those of upper-mantle confined slabs. Tonga (Fig. 2d) exhibits the opposite behaviour. Its velocities for the past 45 Myr do not exceed the range for upper-mantle slab buoyancy.

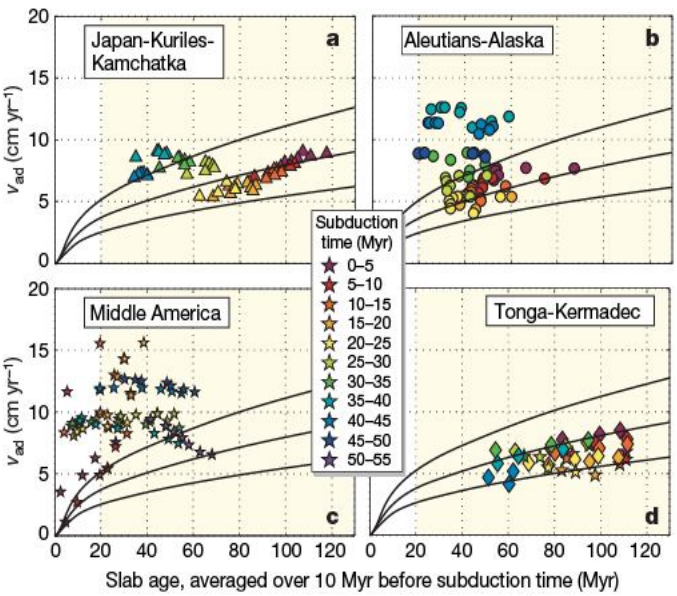

Figure 2 | Past plate-advance velocities. **a–d**, Comparison between Cenozoic advance velocities of the downgoing plate, v_{ad}, versus average upper-mantle slab age, and the upper-mantle slab Stokes velocities that bracket present-day sinking rates (Fig. 1 main panel; probably only self-sustaining above 20 Myr slab age); data are given for selected zones (Fig. 1 inset) shown boxed in each panel. Velocities are shown at points spaced 500 km along the trench, exactly as in ref. 3, because an appropriate segmentation would vary in time. In **d**, different symbols are used for the Tonga (diamonds) and Kermadec (stars) sections of the trench.

There is a trend of velocity with age, specifically for Tonga. Subduction at the Kermadec end of the trench has probably been slowed down by subduction of the Louisville ridge and the Hikurangi plateau.

Interestingly, the phases of the highest excess downgoing-plate velocities coincide with minima in trench velocities, both in absolute sense and relative to subduction velocities (Supplementary Fig. 3), similar to the signature that models display during lower-mantle slab penetration[17,18]. Trench motions in Japan and Alaska reach a minimum of 0.07 times plate-advance. Minimum trench/plate motion ratios at the Middle America trench are slightly higher, 0.1–0.2, but absolute and relative retreat velocities are at least a factor of two lower than between 50 and 55 Myr ago. For Tonga, rollback velocities are generally quite high with phases of active back-arc spreading. We interpret these observations as evidence for past lower-mantle penetration in Japan and Alaska, almost continuous Cenozoic lower-mantle penetration under Middle America, and upper-mantle confined subduction below Tonga for the past 45 Myr.

The inferred lower-mantle penetration phases are compatible with seismic tomography. Material sinking into the lower mantle since 50 or 25 Myr ago, at rates of 1–2 cm yr^{-1}, would have reached a depth of 1,150–1,650 or 900–1,150 km, respectively. The Farallon anomaly below Middle America and the northern part of South America (where some of today's sinking velocities are also quite high; Fig. 1) is strongest down to about 1,700 km depth[5,6], compatible with 50 Myr of continuous slab sinking into the lower mantle. Under South America, where relatively young lithosphere subducted throughout the Cenozoic, periods of very high subduction velocities, coincident with minima in trench retreat, also occur, especially at the northern end[3] (Supplementary Fig. 5). The tomographic models also image high-velocity material in the lower mantle below Japan at least down to about 1,000–1,200 km, possibly deeper. In the upper mantle, the Japan and southern Kurile slabs are flattened above the 660 km discontinuity, and the imaged upper-mantle slab length is similar to the 1,500–2,000 km slab subducted in the past 25 Myr. The amount of flattening and the subduction velocities decrease northwards until lower- and upper-mantle anomalies align under Kamchatka[5,6]. Under Alaska and the Aleutians, upper-mantle tomography away from the trench is not well resolved, but a flat slab seems to be present in the transition zone below the Aleutians[6], while material down to about 1,300 km depth is found below the eastern Aleutians-Alaska[9]. The imaged Tonga slab also flattens in the transition zone and its length can account for the past 45 Myr of subduction. The same holds for Izu-Bonin, where exceedance of upper-mantle subduction velocity trends during the ~45 Myr history of this zone is marginal, and until recently there has been significant trench retreat with active back-arc spreading[3] (Supplementary Fig. 5), compatible with the long, flat-lying upper-mantle slab anomaly[5,6].

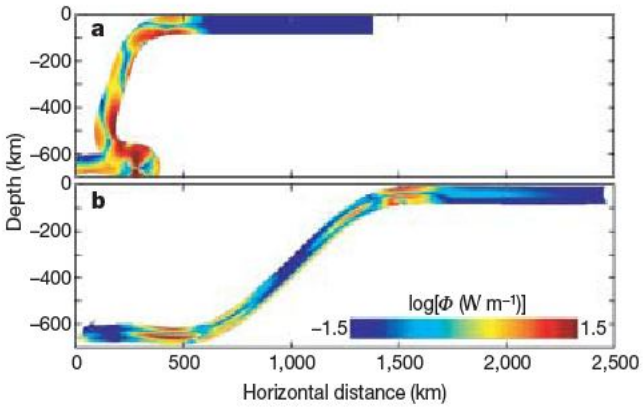

Figure 3 | Model slab deformation. Two models (set up as described in Supplementary Information, details in ref. 4) illustrate how deformation above the upper-mantle/lower-mantle boundary depends on plate properties, for plates subjected to ridge push and a low-drag asthenosphere. In **a**, a plate with buoyancy appropriate for a young downgoing slab (equivalent of 49 kg m^{-3} density times 80 km thickness), with a uniform viscosity of 100 times that of the surrounding mantle, buckles and thickens. In contrast, in **b**, a plate with old-plate buoyancy (88 kg m^{-3} times 80 km), and a strong 17-km-thick core with a viscosity 1,000 times that of the upper mantle (so that average plate viscosity equals 100× upper-mantle viscosity), retreats much more, resulting in a flat lying slab. Both snapshots are after 60% of the plate has been subducted. Colours represent lithospheric energy dissipation (Φ = strain rate × stress) at this point in time, where red regions deform strongly and blue regions deform little.

It may seem surprising that it is the younger, less buoyant, lithosphere that preferentially penetrates quickly into the lower mantle. However, because of its lower buoyancy, freely subducting young lithosphere drives less trench retreat than old lithosphere (Fig. 3). This effect is enhanced if younger lithosphere also has a lower resistance to bending[4]. In addition, zones subducting younger lithosphere seem often unable to initiate back-arc spreading[3,29], which further hampers trench retreat. Low trench retreat and low slab strength both encourage slab deformation in the transition zone (Fig. 3).

If trench retreat is facilitated—for example, by a small slab width, as in the case of Calabria or South Sandwich—even young lithosphere may avoid fast lower-mantle penetration. In contrast, if outside forces are sufficient to hamper or force the motion of a trench that consumes old plate, they may induce lower-mantle penetration. This could be happening at the Marianas trench (which has pivoted and advanced around a point near its southern end[3]) and Kermadec (where buoyant lithosphere impinges on the partially advancing trench). In both cases, a steeply dipping slab anomaly is seen to penetrate down to 1,000 km depth[5,6], although plate-advance velocities are not anomalously high, which may indicate that their lower-mantle penetration is not driven by excess slab buoyancy.

Without thickening, slab Stokes velocities decrease in proportion to the upper-lower mantle viscosity increase, resulting in lower-mantle sinking velocities of at most a few mm yr^{-1}. For a viscosity increase by a factor of 10, slabs would need to thicken 1.4–4 times to attain the 1–2 cm yr^{-1} lower-mantle sinking velocities that reconcile seismic tomography and subduction history. At a subduction rate of 7 cm yr^{-1}, vertically sinking slabs can thicken by that much over a timescale of 14–40 Myr. This timescale is similar to the few tens of Myr over which variations in subduction mode occur in the data (Fig. 2).

Throughout the Cenozoic, subduction of young lithosphere commonly satisfied the conditions of low trench retreat and easy transition-zone thickening, which facilitate free and fast lower-mantle penetration, while old lithosphere usually did not. Such penetration speeds up young plate subduction to rates similar to or higher than the rates at which old plate subducts under the influence of upper-mantle slab pull, thus providing a mechanism that buffers plate motion rates.

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LETTERS

A fundamental avian wing-stroke provides a new perspective on the evolution of flight

Kenneth P. Dial[1], Brandon E. Jackson[1] & Paolo Segre[1]

The evolution of avian flight remains one of biology's major controversies, with a long history of functional interpretations of fossil forms given as evidence for either an arboreal or cursorial origin of flight. Despite repeated emphasis on the 'wing-stroke' as a necessary avenue of investigation for addressing the evolution of flight[1–4], no empirical data exist on wing-stroke dynamics in an experimental evolutionary context. Here we present the first comparison of wing-stroke kinematics of the primary locomotor modes (descending flight and incline flap-running) that lead to level-flapping flight in juvenile ground birds throughout development (Fig. 1). We offer results that are contrary both to popular perception and inferences from other studies[5–7]. Starting shortly after hatching and continuing through adulthood, ground birds use a wing-stroke confined to a narrow range of less than 20°, when referenced to gravity, that directs aerodynamic forces about 40° above horizontal, permitting a 180° range in the direction of travel. Based on our results, we put forth an ontogenetic-transitional wing hypothesis that posits that the incremental adaptive stages leading to the evolution of avian flight correspond behaviourally and morphologically to transitional stages observed in ontogenetic forms.

Just as evolutionary developmental biology is providing remarkable advances in our understanding of the history of organismal diversity and construction of body plans, we propose that explorations of the ontogeny of post-natal behaviour and morphology among extant taxa provide insight into ecological and evolutionary locomotor transitional stages. With this perspective, we studied the locomotor development of hatchling to adult chukars (*Alectoris chukar*), a common ground bird. Here we focus on two critical variables that define the orientation of the resultant aerodynamic vector

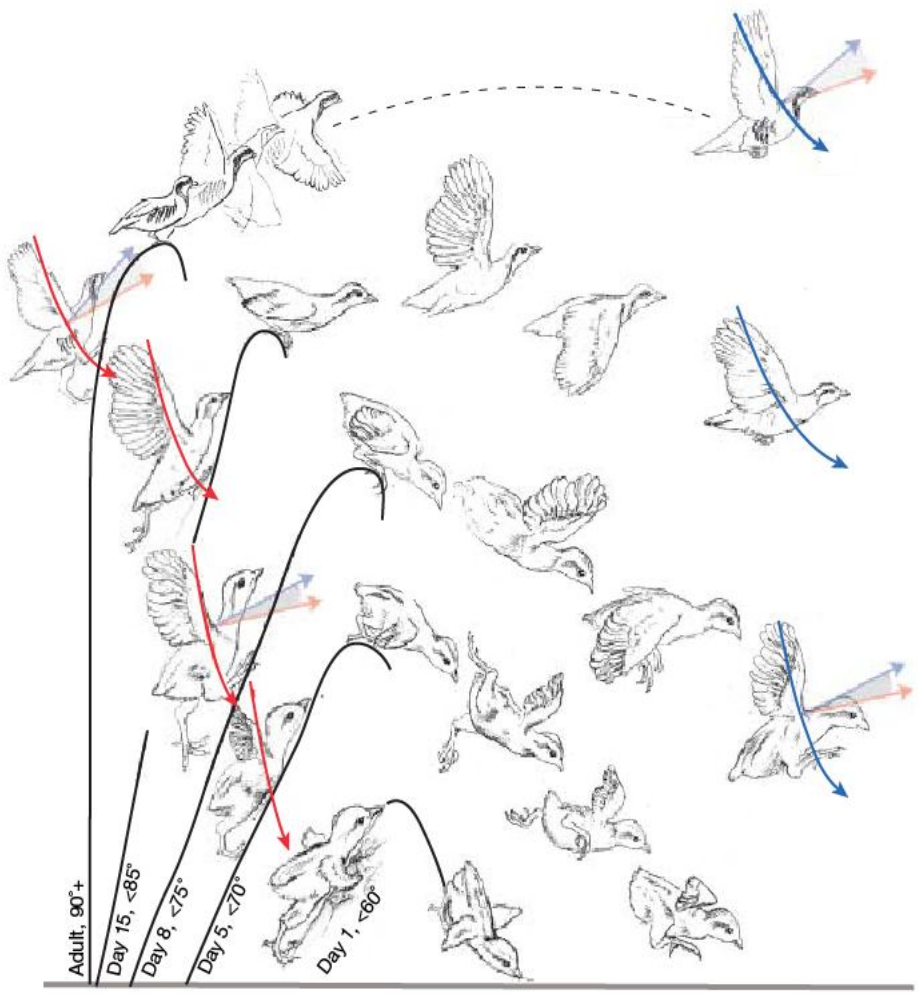

Figure 1 | Locomotor development during ontogeny in the chukar partridge from hatching to adulthood. Our data suggest a default or basal wing-stroke is used by young and adults and may exist in all birds (Supplementary Videos). The fundamental wing-stroke described herein is used days after hatching and during all ages and over multiple behaviours (that is, flap-running, descending and level flight) and is the foundation of our new ontogenetic-transitional wing hypothesis. At hatching, chicks can ascend inclines as steep as 60° by crawling on all four limbs. From day 8 through adulthood, birds use a consistently orientated stroke-plane angle over all substrate inclines during wing-assisted incline running (red arcs) as well as during descending and level flight (blue arcs). Estimated force orientations from this conserved wing-stroke are limited to a narrow wedge (see Fig. 3b).

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during flapping: (1) stroke-plane angle (SPA, the angle of the plane swept out by the wing in downstroke); and (2) angle of attack (AOA, the angle of the wing-plane). To provide a rigorous experimental investigation, we compare three-dimensional (3D) kinematics during level flapping flight (the definitive stage of aerial locomotor capacity in avian evolution) with the primary locomotor behaviours involving forelimb use that precede level flight in developing ground birds, (1) flap-descending flight and (2) flap running (also called wing-assisted incline running, WAIR[8–11]), over a wide-range of inclines. These experimental conditions and ontogenetic stages represent a broad continuum of wing morphologies and locomotor behaviours that are exhibited by birds.

Analysing our data from the traditional vertebral frame of reference (that is, relative to the body axis, Supplementary Fig. 1), we expected and found that in all age classes, the vertebral SPA transitioned (greater than 30°) from a relatively anterior–posterior orientation during incline flap-running (WAIR), through dorso-ventral, to a slightly posterior–anterior orientation in flight (Figs 2a and 3a). Consequently, the vertebral AOA (the amount of pronation or supination of the wing with respect to the body) at the midpoint of downstroke shifted at least 45° from strongly pronated in WAIR to nearly parallel to the vertebral axis in flight (Table 1). The SPA and AOA results could be interpreted prima facie as aerodynamic forces acting in different directions (that is, ventrally in WAIR, dorso-anteriorly in flight) and support the observation that birds substantially change their wing-stroke when executing different behaviours. Coincidentally, most historical reconstructions of the origin of the wing-stroke and avian flight (for review, see refs 12 and 13) rely on the vertebral axis to describe forelimb transitional stages over evolutionary time, which has impeded the development of alternative hypotheses.

Our results led us to consider alternative frames of reference[14] that allow an evaluation of the function of flapping wings (whether proto- or flight-capable wings), to generate aerodynamic forces primarily to overcome gravitational forces and thus offer weight support. Therefore, we examined kinematics in two external frames of reference (global and gravitational, Supplementary Fig. 1 and Supplementary Methods). Briefly, the global frame of reference allows evaluation of aerodynamic force orientation whereas the gravitational frame of reference allows an evaluation of wing kinematics relative to gravity while accounting for the movement of the bird's body. Here we unexpectedly found SPA and AOA to be remarkably similar among vastly different locomotor behaviours (Figs 2b, c and 3, Table 1 and Supplementary Video 1). Despite the disparate orientations of travel, the estimated orientation of aerodynamic force (orthogonal to the global SPA) fell within a narrow wedge (19°, Fig. 3b). Juveniles began to exhibit a wing-stroke similar to the adults in SPA and AOA around 8 days post-hatching (Table 1, Supplementary Fig. 2 and Supplementary Videos 2 and 3). This constricted range of aerodynamic force orientation and AOA (Fig. 3c) allows a wide range of locomotor activities in juveniles and adults (Fig. 1) and strongly suggests a stereotypic or fundamental wing-stroke that we hypothesize to be functionally aligned to gravity.

This fundamental wing-stroke does not appear to be unique to the chukars or ground birds studied here. Numerous high-speed video observations by our laboratory of more than 20 avian species

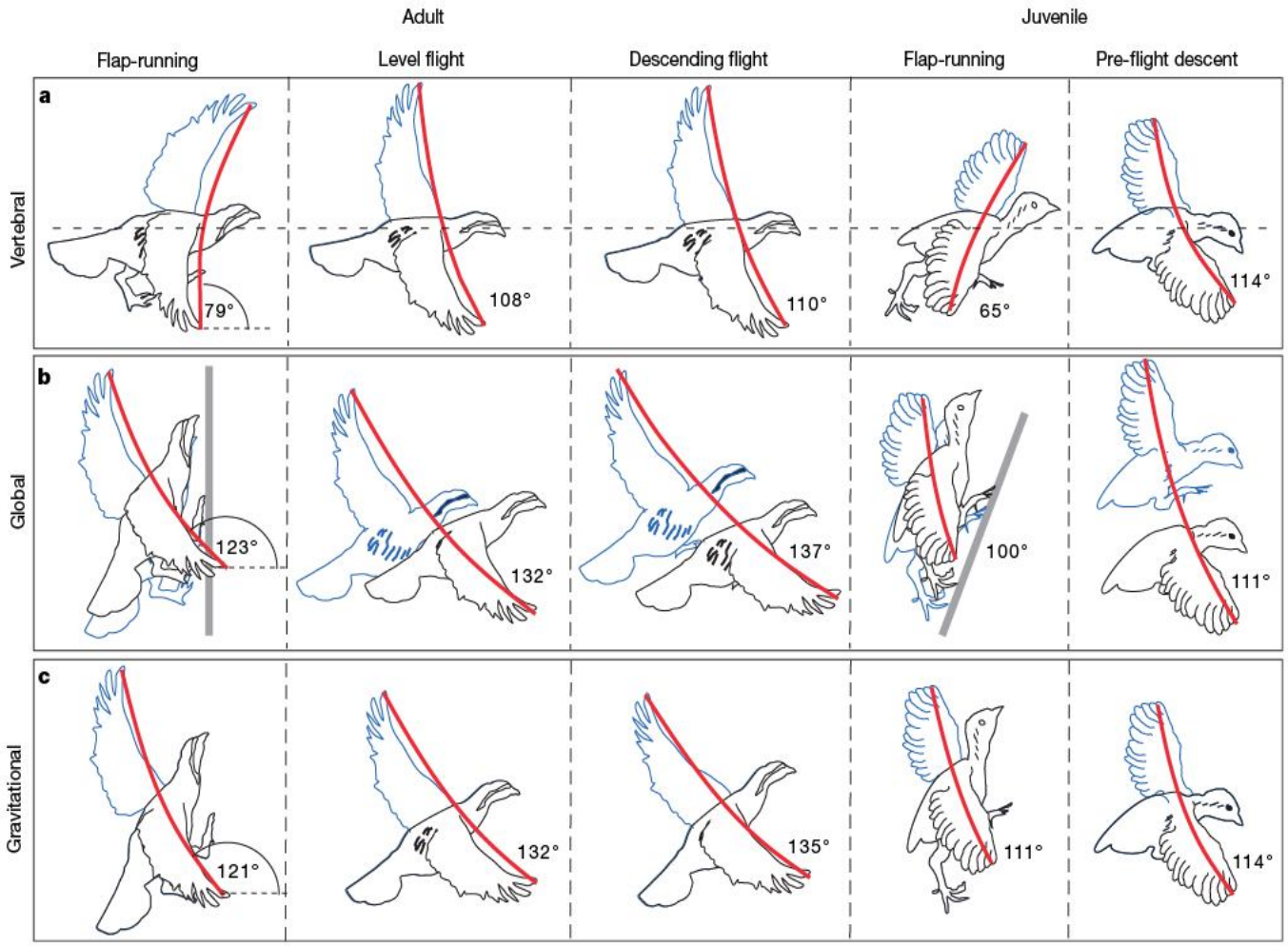

Figure 2 | Average stroke-plane angle among locomotor styles in three frames of reference. Blue and black outlines represent the positions of the bird and wing at the start and end of downstroke, respectively. **a**, In the vertebral space, the mean wing-stroke plane angle shifts more than 30° from a more antero-posterior orientation during flap-running to dorso-ventrally in flight, implying different wing-strokes are used to execute different locomotor modes. The wing-stroke path is consistently oriented, however, in both the (**b**) global and (**c**) gravitational coordinate spaces over diverse locomotor behaviours, illustrating a simplified wing-stroke that is multi-functional. Data for juveniles are presented from 8- to 10-day olds.

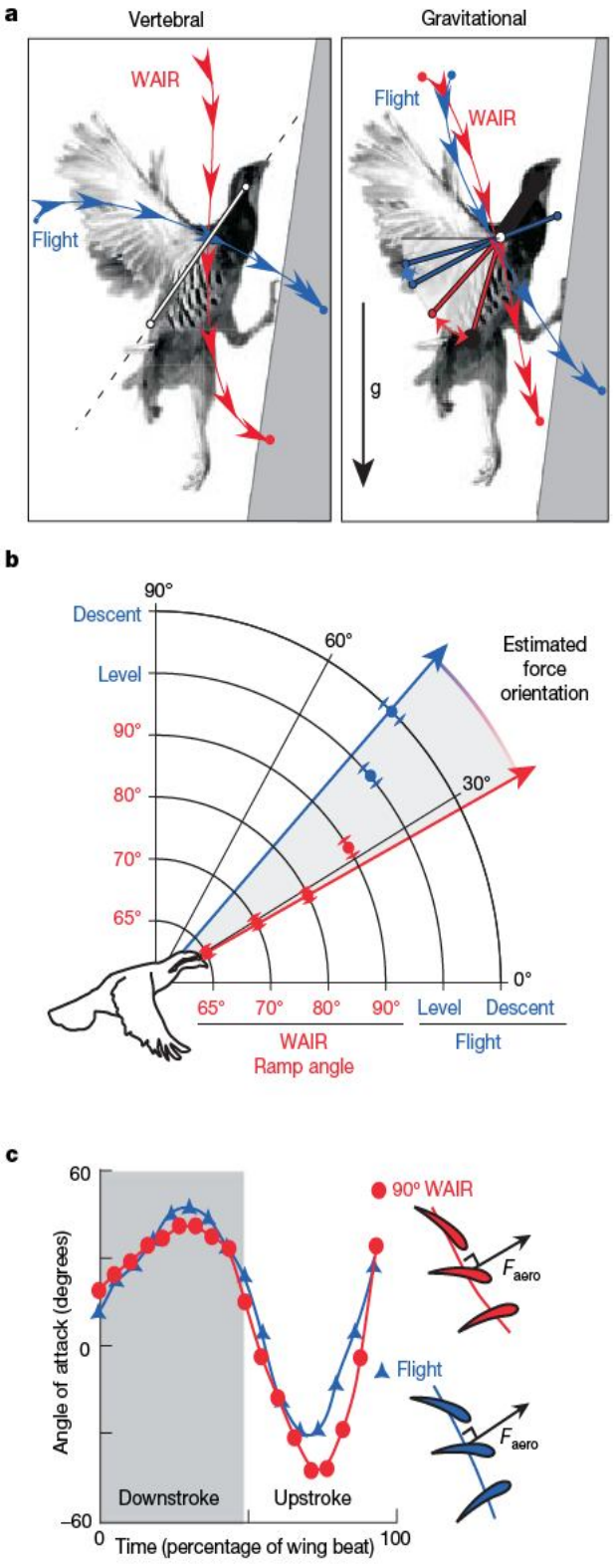

Figure 3 | Comparison of wing-stroke plane angle, estimated force orientation and angle of attack among locomotor styles. **a**, When wing-stroke plane angles are viewed side-by-side in both the vertebral and gravitational frames of reference, the wing-stroke is nearly invariant relative to gravity whereas the body axis re-orients among different modes of locomotion. Red lines represent the wing-tip trace in WAIR (flap-running) and blue lines represent the wing-tip trace in level flight. **b**, Wing-strokes are estimated to produce similar aerodynamic forces oriented about 40° above the horizon during WAIR, level flight and descending flight. Error bars are s.e.m. **c**, Representative traces of AOA through a wing-beat for an animal flap-running vertically (red) and in horizontal flight (blue) demonstrate the similarities of AOA among behaviours. The similarities are further clarified by examining wing cross-sections and mean global stroke-planes in the first, middle and last thirds of downstroke. Here, the orientation of the aerodynamic force (F_{aero}) is estimated from the middle third.

(ranging from basal, for example Tinamiformes, to derived, for example Passeriformes; Supplementary Video 4) demonstrate that most taxa are capable of similar body axis to wing-stroke postures (during WAIR and free flight). This suggests that the fundamental wing-stroke we describe is plesiomorphic and elementary to understanding critical elements of avian locomotion, and perhaps its evolution.

We propose that this fundamental wing-stroke provides sufficient locomotor capacity for many basal taxa (for example, Tinamiformes, Galliformes, Anseriformes[15]) that have relatively fixed flight speeds and narrow flight styles. The range of locomotor behaviours exhibited by basal birds is accomplished by adjusting power output (estimated here from WBF and stroke amplitude; Table 1) consistent with changes in locomotor demand while slinging the body to optimal positions around the stroke-plane. Analogously, helicopters accomplish a wide range of locomotor capacity within a similarly restricted wedge of rotor plane angles by modifying power output and slinging the body of the aircraft around beneath the rotor-plane. Derived taxa (for example, Falconiformes, Columbiformes, Psittaciformes, Passeriformes) are expected to use this fundamental wing-stroke yet are capable of modifying wing excursion to allow advanced forms of aerial locomotion (for example, an array of flight speeds, which makes them more tractable for variable-speed wind-tunnel studies, more precise landings and superior manoeuvrability[16,17]). Yet its existence in basal taxa also provides a key inference for the evolution of flight.

The general impression (including those of the authors, originally) that birds change their wing-stroke to execute different locomotor behaviours stems from casual observation of birds in the field and is reinforced by inferences generated by quantitative kinematic studies of derived taxa during flapping flight in variable-speed wind tunnels working in the vertebral frame of reference[5–7]. Such studies accurately delineate changes in wing-stroke and body angle as birds match air speeds. In this study we allowed the birds to choose their preferred locomotor speed, as they do in natural settings and in contrast to wind-tunnel flight, we did so over a range of flapping behaviours, and we used multiple frames of reference. This alternative approach demonstrated that basal birds exhibit a relatively fixed wing-stroke and alter power to achieve differing locomotor behaviours.

The fact that this relatively fixed wing-stroke is expressed at several days post-hatching (Supplementary Fig. 2) raises the question, what function does it serve before the fledgling can achieve level flight? We now know that very young birds possessing only partly developed wings are able to produce significant and functional aerodynamic forces (even with their symmetrically constructed feathers[10,11], contrary to published comments[18–23]); these forces assist them in climbing to an elevated refuge[11] and when they descend to a lower substrate resulting in a lower impact speed[24]. Thus, the wing-stroke and a proto-wing have a function early in life to negotiate immediately 3D terrestrial habitats and ultimately the aerial environment. If extant flight-incapable bipeds are able to enjoy incremental aerodynamic contributions from flapping developing wings, we argue that proto-bird ancestors lacking flight-capable forelimbs may also have done so (Fig. 1).

Based on our results, we put forth an ontogenetic-transitional wing (OTW) hypothesis for the origin of flight. The hypothesis posits that the transitional stages leading to the evolution of avian flight correspond both behaviourally and morphologically to the transitional stages observed in ontogenetic forms. Specifically, from flightless hatchlings to flight-capable juveniles, many ground birds express a 'transitional wing' during development that is representative of evolutionary transitional forms. Our experimental observations reveal that birds move their 'proto-wings', and their fully developed wings, through a stereotypic or fundamental kinematic pathway so that they may flap–run over obstacles[8–11], control descending flight[24] and ultimately perform level flapping flight (Fig. 1). The OTW hypothesis provides a simple adaptive argument for the evolution of flight

Table 1 | Summary statistics for key three-dimensional kinematic variables

Kinematic variable	WAIR						Level flight	Descending flight		
	65°		70°	80°	90°	F‡		Adult	Juvenile	F§
	Adult	Juvenile								
N (wing beats)	15	13	11	18	18		10	24	5	
Body angle (deg.)†	55 (3)	43 (2)	58 (2)	66 (2)	65 (4)	2.99**	47 (7)	47 (4)	−28 (12)	6.63*
Wing beat frequency (Hz)	17.2 (0.5)	19.3 (1.0)	18.0 (0.8)	19.3 (0.1)	19.9 (0.2)	10.27**	22.7 (0.1)	20.2 (0.2)	28.6 (2)	122.01**
Stroke amplitude (deg.)	136 (3)	144 (7)	149 (6)	151 (4)	149 (3)	3.20*	127 (7)	118 (5)	111 (23)	13.28*
Angle of attack (deg.)	35 (1)	50 (6)	34 (2)	27 (2)	28 (2)	3.98*	30 (2)	33 (1)	42 (14)	0.01
Stroke-plane angle (deg.)										
Vertebral	82 (3)	65.4 (4)	78 (2)	78 (2)	79 (3)	0.50	108 (2)	110 (2)	104 (7)	48.70**
Global	120 (1)	94 (3)	119 (2)	118 (2)	123 (2)	1.45	132 (2)	137 (2)	105 (13)	12.34*
Gravitational	118 (1)	91 (3)	116 (2)	116 (2)	121 (2)	2.29	132 (2)	135 (5)	94 (9)	13.23*

* $P < 0.05$; ** $P < 0.001$. †All data presented as mean (s.e.m.). ‡Repeated-measures analysis of variance, factor: ramp angle (adult data). §Repeated-measures analysis of variance, factor: 90° ramp versus level flight (adult data).

and can be tested and observed in extant fledglings. This hypothesis differs from other published accounts in that it is flap-based (in contrast to requiring a gliding precursor), involves an aerodynamically functional proto-wing[11], incorporates both the simultaneous and independent use of legs and wings[8–10] and assumes that a fundamental wing-stroke (described herein) was established for aerodynamic function early in the bipedal ancestry leading to birds. Such an evolutionary pathway provides a parsimonious explanation for numerous non-avian theropod morphologies (for example, semilunate carpal, delto-pectoral crest, furcula, proto-wings, symmetrically vaned feathers, long bipedal hindlimbs, etc.) that have not been discussed in a synthetic context.

The unequivocal morphological changes in the shoulder during the evolution of birds[25,26] are compatible with the OTW hypothesis. The shoulder joint (glenoid) is thought to have evolved from a primitive ventro-lateral orientation allowing a cranial–caudal excursion (as observed in theropod ancestors) to the derived dorso-lateral orientation allowing a dorso-ventral excursion (among extant flying birds)[26]. Jenkins[26] suggested the 90° rotation of the glenoid's excursion axis relative to the body was to accommodate the derived wing-stroke angle of extant birds. We agree with the character states Jenkins eloquently describes and offer a novel perspective about the process underlying the evolutionary sequence. We suggest the orientation of the shoulder joint remained relatively fixed in 3D space (in the global and gravitational frames of reference) over evolutionary time. This allowed the body axis to rotate, up to 90°, resulting in the observed character states of the shoulder (in the vertebral frame of reference), described above. Living ground birds exhibit a slinging of the torso about the shoulder (Fig. 3a). We suggest this same feature allowed proto-birds to use a functional wing-stroke (even with proto-wings) aligned to gravity which assisted their hindlimbs as they scaled increasingly pitched obstacles, allowed controlled flapping descent and powered rudimentary flight in the transitional stages leading to level flapping flight (Fig. 1). In other words, the gravity-based wing-stroke did not come about through a long series of migrational stages of the forelimb (from ventro-lateral to lateral to dorso-lateral): rather, the primitive wing-stroke started in a similar orientation as we see it today in hatchlings using their proto-wings.

Perhaps we can cut the Gordian knot created by the false dichotomy[4] of the highly charged, but unresolved, cursorial–arboreal debate. The OTW hypothesis embraces salient features of both the arboreal and cursorial hypotheses yet clearly differs from both. For example, arboreal hypotheses assume a gliding form was prerequisite to flapping flight because half a wing would have no function, and that the flap-stroke appears too complex and thus relegated to the derived condition. However, this line of reasoning is inconsistent with observations of all extant forms. For example, gliding and soaring are essentially absent in the most basal avian clades (that is, Tinamiformes, Galloanserae[15]) as well as in early ontogenetic stages of all birds: these forms flap their forelimbs. We propose gliding to be the derived condition within Aves because it is mostly confined to adult-sized individuals of non-basal taxa. A serious flaw with the assumption of a gliding precursor transitioning towards flapping is the fact that not a single species, among hundreds of living non-avian vertebrate gliders, flaps their webbed appendages to generate powered thrust or lift. Commonly held assumptions within the cursorial school about the plausible function of proto-wings are inconsistent with the ontogenetic biology of extant forms; for example, no extant species uses its wings to run faster, to secure prey or run–glide.

Locomotor abilities of extinct taxa, such as the recently discovered fossil forms possessing what is assumed to be 'half a wing'[27,28] and long cursorial legs[29], might be better understood if we evaluate how proto-wings and hindlimbs function during ontogeny in extant taxa[8,10]. Our experimental observations show that proto-wings moving through a stereotypic and conserved wing-stroke have immediate aerodynamic function, and that transitioning to powered flapping flight is limited by the relative size of the wing and muscle power, rather than development of a complex repertoire of wing-beat kinematics.

METHODS SUMMARY

We used four internally synchronized high-speed digital video cameras (250 Hz, 1250 s^{-1}) to record chukars every two days, beginning one day after hatching through to adulthood, while they passed through a 3D calibrated volume. We quantified 12 kinematic variables (wing beat frequency, duty factor, body and wing velocities, wing angular velocity, body angle, stroke amplitude, stroke plane angle, angle of attack, dynamic wing loading, dynamic wing length and actuator disc loading) in 3D space to characterize body and wing dynamics (Supplementary Fig. 1). Ten points on the right wing and body were marked with reflective tape, digitized and analysed by direct linear transformation in Ariel Performance Analysis Software (Ariel Dynamics, Inc.). Computations of vectors, planes and angles were performed in a custom program within IGOR Pro (WaveMetrics, Inc.).

Full Methods and any associated references are available in the online version of the paper at www.nature.com/nature.

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LETTERS

Global trends in emerging infectious diseases

Kate E. Jones[1], Nikkita G. Patel[2], Marc A. Levy[3], Adam Storeygard[3]†, Deborah Balk[3]†, John L. Gittleman[4] & Peter Daszak[2]

Emerging infectious diseases (EIDs) are a significant burden on global economies and public health[1–3]. Their emergence is thought to be driven largely by socio-economic, environmental and ecological factors[1–9], but no comparative study has explicitly analysed these linkages to understand global temporal and spatial patterns of EIDs. Here we analyse a database of 335 EID 'events' (origins of EIDs) between 1940 and 2004, and demonstrate non-random global patterns. EID events have risen significantly over time after controlling for reporting bias, with their peak incidence (in the 1980s) concomitant with the HIV pandemic. EID events are dominated by zoonoses (60.3% of EIDs): the majority of these (71.8%) originate in wildlife (for example, severe acute respiratory virus, Ebola virus), and are increasing significantly over time. We find that 54.3% of EID events are caused by bacteria or rickettsia, reflecting a large number of drug-resistant microbes in our database. Our results confirm that EID origins are significantly correlated with socio-economic, environmental and ecological factors, and provide a basis for identifying regions where new EIDs are most likely to originate (emerging disease 'hotspots'). They also reveal a substantial risk of wildlife zoonotic and vector-borne EIDs originating at lower latitudes where reporting effort is low. We conclude that global resources to counter disease emergence are poorly allocated, with the majority of the scientific and surveillance effort focused on countries from where the next important EID is least likely to originate.

In the global human population, we report the emergence of 335 infectious diseases between 1940 and 2004. Here we define the first temporal origination of an EID (that is, the original case or cluster of cases representing an infectious disease emerging in human populations for the first time—see Methods and Supplementary Table 1) as an EID 'event'. Our database includes EID events caused by newly evolved strains of pathogens (for example, multi-drug-resistant tuberculosis and chloroquine-resistant malaria), pathogens that have recently entered human populations for the first time (for example, HIV-1, severe acute respiratory syndrome (SARS) coronavirus), and pathogens that have probably been present in humans historically, but which have recently increased in incidence (for example, Lyme disease). The emergence of these pathogens and their subsequent spread have caused an extremely significant impact on global health and economies[1–3]. Previous efforts to understand patterns of EID emergence have highlighted viral pathogens (especially RNA viruses) as a major threat, owing to their often high rates of nucleotide substitution, poor mutation error-correction ability and therefore higher capacity to adapt to new hosts, including humans[5,8,10,11]. However, we find that the majority of pathogens involved in EID events are bacterial or rickettsial (54.3%). This group is typically represented by the emergence of drug-resistant bacterial strains (for example, vancomycin-resistant *Staphylococcus aureus*). Viral or prion pathogens constitute only 25.4% of EID events, in contrast to previous analyses which suggest that 37–44% of emerging pathogens are viruses or prions and 10–30% bacteria or rickettsia[5,8,11]. This follows our classification of each individual drug-resistant microbial strain as a separate pathogen in our database, and reflects more accurately the true significance of antimicrobial drug resistance for global health, in which different pathogen strains can cause separate significant outbreaks[12]. In broad concurrence with previous studies on the characteristics of emerging human pathogens[5,8,11], we find the percentages of EID events caused by other pathogen types to be 10.7% for protozoa, 6.3% for fungi and 3.3% for helminths (see Supplementary Data and Supplementary Table 2 for a detailed comparison to previous studies).

The incidence of EID events has increased since 1940, reaching a maximum in the 1980s (Fig. 1). We tested whether the increase through time was largely attributable to increasing infectious disease reporting effort (that is, through more efficient diagnostic methods and more thorough surveillance[2,3,13]) by calculating the annual number of articles published in the *Journal of Infectious Diseases* (JID) since 1945 (see Methods). Controlling for reporting effort, the number of EID events still shows a highly significant relationship with time (generalized linear model with Poisson errors, offset by log(JID articles) ($GLM_{P,JID}$), $F = 96.4$, $P < 0.001$, d.f. = 57). This provides the first analytical support for previous suggestions that the threat of EIDs to global health is increasing[1,2,14]. To further investigate the peak in EID events in the 1980s, we examined the most frequently cited driver of EID emergence during this period (see Supplementary Table 1). Increased susceptibility to infection caused the highest proportion of events during 1980–90 (25.5%), and we therefore suggest that the spike in EID events in the 1980s is due largely to the emergence of new diseases associated with the HIV/AIDS pandemic[2,13].

The majority (60.3%) of EID events are caused by zoonotic pathogens (defined here as those which have a non-human animal source), which is consistent with previous analyses of human EIDs[5,8]. Furthermore, 71.8% of these zoonotic EID events were caused by pathogens with a wildlife origin—for example, the emergence of Nipah virus in Perak, Malaysia and SARS in Guangdong Province, China. The number of EID events caused by pathogens originating in wildlife has increased significantly with time, controlling for reporting effort ($GLM_{P,JID}$ $F = 60.7$, $P < 0.001$, d.f. = 57), and they constituted 52.0% of EID events in the most recent decade (1990–2000) (Fig. 1). This supports the suggestion that zoonotic EIDs represent an increasing and very significant threat to global health[1,2,7,13,14]. It also highlights the importance of understanding the factors that increase contact between wildlife and humans in developing predictive approaches to disease emergence[4,6,9,15].

Vector-borne diseases are responsible for 22.8% of EID events in our database, and 28.8% in the last decade (Fig. 1). Our analysis

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reveals a significant rise in the number of EID events they have caused over time, controlling for reporting effort ($GLM_{P,JID}$ $F = 49.8$, $P < 0.001$, d.f. = 57). This rise corresponds to climate anomalies occurring during the 1990s[16], adding support to hypotheses that climate change may drive the emergence of diseases that have vectors sensitive to changes in environmental conditions such as rainfall, temperature and severe weather events[17]. However, this controversial issue requires further analyses to test causal relationships between EID events and climate change[18]. We also report that EID events caused by drug-resistant microbes (which represent 20.9% of the EID events in our database) have significantly increased with time, controlling for reporting effort ($GLM_{P,JID}$ $F = 5.19$, $P < 0.05$, d.f. = 57). This is probably related to a corresponding rise in antimicrobial drug use, particularly in high-latitude developed countries[2,7,12].

A recent analysis showed a latitudinal spatial gradient in human pathogen species richness increasing towards the Equator[19], in common with the distributional pattern of species richness found in many other taxonomic groups[20]. Environmental parameters that promote pathogen transmission at lower latitudes (for example, higher temperatures and precipitation) are hypothesized to drive this pattern[19]. Our analyses suggest that there is no such pattern in EID events, which are concentrated in higher latitudes (Supplementary Fig. 1). The highest concentration of EID events per million square kilometres of land was found between 30 and 60 degrees north and between 30 and 40 degrees south, with the main hotspots in the northeastern United States, western Europe, Japan and southeastern Australia (Fig. 2). We hypothesize that (1) socioeconomic drivers (such as human population density, antibiotic drug use and agricultural practices) are major determinants of the spatial distribution of EID events, in addition to the ecological or environmental conditions that may affect overall (emerging and non-emerging) human pathogen distribution[19], and (2) that the importance of these drivers depends on the category of EID event. In particular, we hypothesize that EID events caused by zoonotic pathogens from wildlife are significantly correlated with wildlife biodiversity, and those caused by drug-resistant pathogens are more correlated with socio-economic conditions than those caused by zoonotic pathogens.

We tested these hypotheses by examining the relationship between the spatial pattern of the different categories of EID events (zoonotic pathogens originating in wildlife and non-wildlife, drug-resistant and vector-borne pathogens, Supplementary Fig. 2), and socioeconomic variables (human population density and human population growth), environmental variables (latitude, rainfall) and an ecological variable (wildlife host species richness) (see Methods). We found that human population density was a common significant independent predictor of EID events in all categories, controlling for spatial reporting bias by country (see Methods, Table 1 and Supplementary Table 3). This supports previous hypotheses that disease emergence is largely a product of anthropogenic and demographic changes, and is a hidden 'cost' of human economic development[2,4,7,9,13]. Wildlife host species richness is a significant predictor for the emergence of zoonotic EIDs with a wildlife origin, with no role for human population growth, latitude or rainfall (Table 1). The emergence of zoonotic EIDs from non-wildlife hosts is predicted by human population density, human population growth, and latitude, and not by wildlife host species richness. EID events caused by drug-resistant microbes are affected by human population density and growth, latitude and rainfall. The pattern of EID events caused by vector-borne diseases was not correlated with any of the environmental or ecological variables we examined, although we note that the climate variable used in this analysis (rainfall) does not represent climate change phenomena.

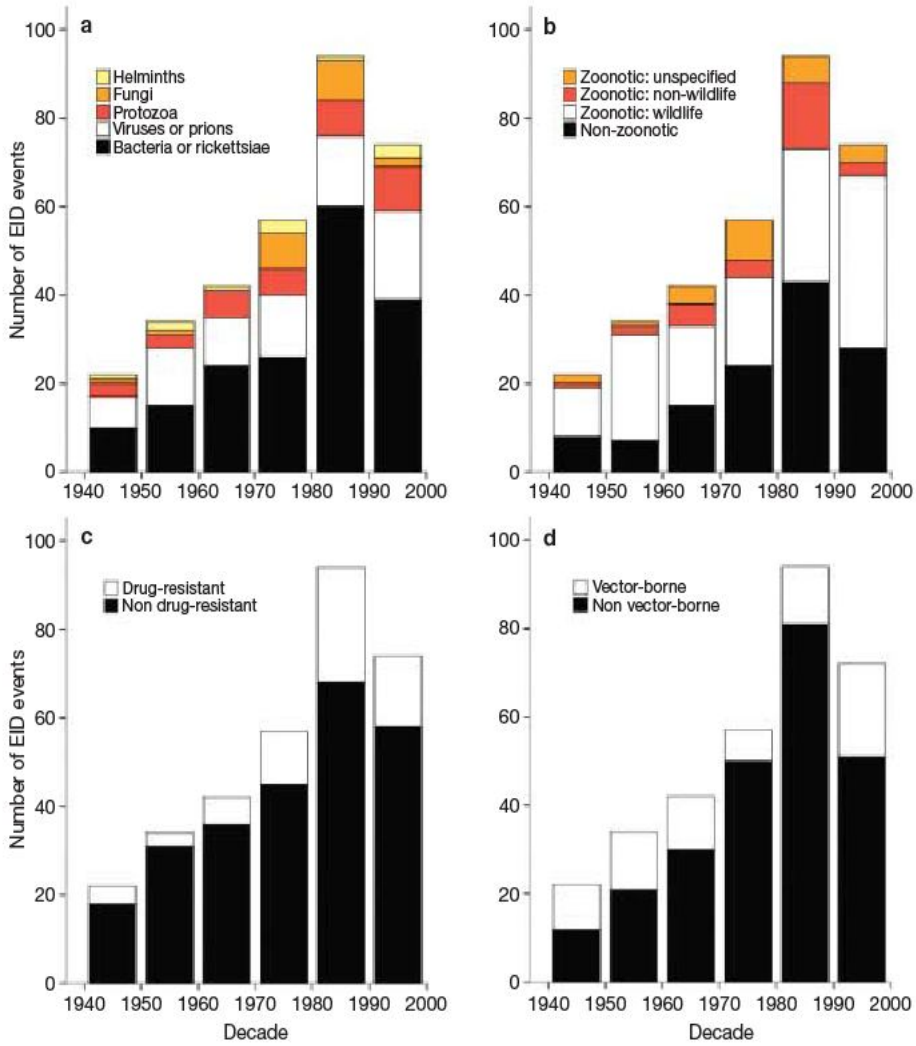

Figure 1 | Number of EID events per decade. EID events (defined as the temporal origin of an EID, represented by the original case or cluster of cases that represents a disease emerging in the human population—see Methods) are plotted with respect to **a**, pathogen type, **b**, transmission type, **c**, drug resistance and **d**, transmission mode (see keys for details).

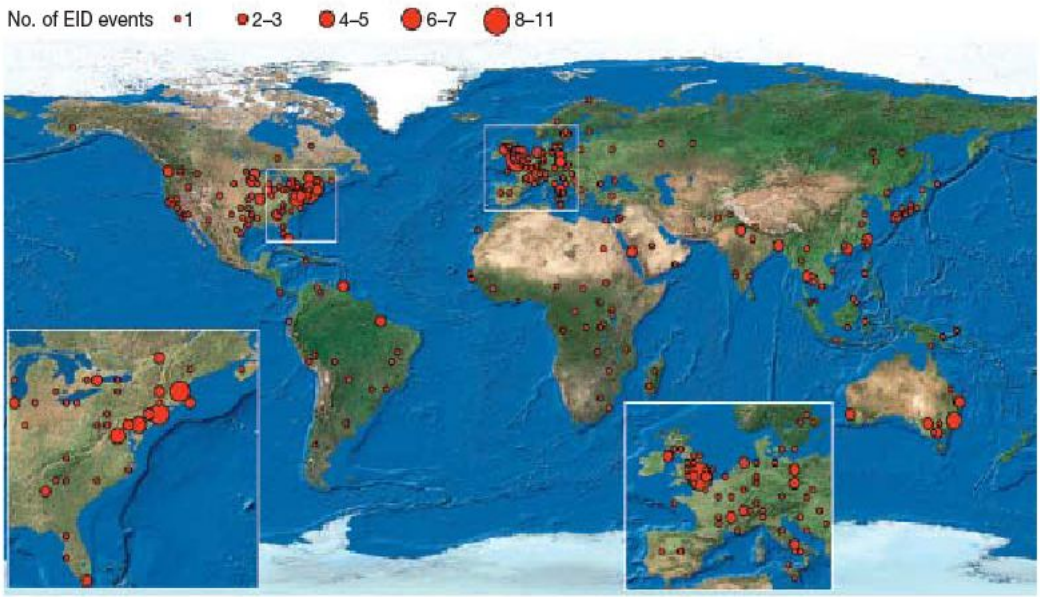

Figure 2 | Global richness map of the geographic origins of EID events from 1940 to 2004. The map is derived for EID events caused by all pathogen types. Circles represent one degree grid cells, and the area of the circle is proportional to the number of events in the cell.

Our study examines the role of only a few drivers to understand disease emergence, whereas many other factors (for example, land use change, agriculture) have been causally linked to EIDs[6,21]. However, until more rigorous global data sets of these drivers become available, data on human population density and growth act as reasonable proxies for such anthropogenic changes. Other likely future improvements to the model would include a more accurate accounting for temporal and spatial ascertainment biases—for example, the development of global spatial data sets of the amount of funding per capita for infectious disease surveillance.

Our analyses provide a basis for developing a predictive model for the regions where new EIDs are most likely to originate (emerging disease 'hotspots'). A visualization of the regression results from Table 1 for EID events from each category (Fig. 3) identifies these regions globally. This approach may be valuable for deciding where to allocate global resources to pre-empt, or combat, the first stages of disease emergence[10,14,18,22]. Our analysis shows that there is a high spatial reporting bias for EID events (see Methods, Supplementary Fig. 3), reflecting greater surveillance and infectious disease research effort in richer, developed countries of Europe, North America, Australia and some parts of Asia, than in developing regions. This contrasts with our risk maps (Fig. 3), which suggest that predicted emerging disease hotspots due to zoonotic pathogens from wildlife and vector-borne pathogens are more concentrated in lower-latitude developing countries. We conclude that the global effort for EID surveillance and investigation is poorly allocated, with the majority of our scientific resources focused on places from where the next important emerging pathogen is least likely to originate. We advocate re-allocation of resources for 'smart surveillance' of emerging disease hotspots in lower latitudes, such as tropical Africa, Latin America and Asia, including targeted surveillance of at-risk people to identify early case clusters of potentially new EIDs before their large-scale emergence. Zoonoses from wildlife represent the most significant, growing threat to global health of all EIDs (see our data in Fig. 1, and recent reviews[1,2,5,8,9,13,14]). Our findings highlight the critical need for health monitoring[4,14,23] and identification of new, potentially zoonotic pathogens in wildlife populations, as a forecast measure for EIDs. Finally, our analysis suggests that efforts to conserve areas rich in wildlife diversity by reducing anthropogenic activity may have added value in reducing the likelihood of future zoonotic disease emergence.

Table 1 | Socio-economic, environmental and ecological correlates of EID events

Pathogen type	Zoonotic: wildlife		Zoonotic: non-wildlife	
Number of EID event grid cells	147–156		49–53	
	b	*B*	*b*	*B*
log(JID articles)	0.34–0.37***	1.41–1.45	0.40–0.49***	1.49–1.63
log[human pop. density (persons per km^2)]	0.56–0.64***	1.75–1.90	0.88–1.06***	2.41–2.89
Human pop. growth (change in persons per km^2,1990–2000)†	0.09–0.45	1.09–1.56	0.86–1.31**	2.37–3.71
Latitude (decimal degrees)	0.002–0.017	1.00–1.02	0.024–0.040*	1.02–1.04
Rainfall (mm)	(0.14–0.06) x 10^{-3}	1.00–1.00	(0.32–0.57) x 10^{-3}#	1.00–1.00
Wildlife host richness	0.008–0.013**	1.01–1.01	−0.015 to −0.003	0.99–1.00
Constant	−9.81 to −8.78***		−13.84 to −11.73***	

Pathogen type	Drug-resistant		Vector-borne	
Number of EID event grid cells	59–64		81–88	
	b	*B*	*b*	*B*
log(JID articles)	0.46–0.53***	1.62–1.71	0.17–0.21***	1.18–1.23
log[human pop. density (persons per km^2)]	1.03–1.27***	2.87–3.92	0.41–0.49***	1.51–1.63
Human pop. growth (change in persons per km^2, 1990–2000)†	1.21–1.70***	2.73–5.06	−0.08 to 0.31	0.93–1.37
Latitude (decimal degrees)	0.047–0.072**	1.04–1.07	−0.015 to 0.002	0.98–1.00
Rainfall (mm)	(0.35–0.61) x 10^{-3}*	1.00–1.00	(0.10–0.28) x 10^{-3}	1.00–1.00
Wildlife host richness	(−0.01 to 0.16) x 10^{-2}	1.00–1.02	(0.28–0.74) x 10^{-2}	1.00–1.01
Constant	−17.45 to −14.41***		−8.21 to −7.53***	

Columns represent multivariable logistic regressions for EID events split according to the type of pathogen responsible. Numbers represent the range of values obtained from 10 random draws of the possible grid squares, where *b* represents the regression coefficients and *B* represents the odds ratio for the independent variables in the model. Higher odds ratios indicate that variable value increases have a higher likelihood of being associated with an EID event; probability value equals the median probability from 10 random draws of the possible grid squares where ***$P < 0.001$, **$P < 0.01$, *$P < 0.05$, #$P < 0.1$. Results from each random draw are shown in Supplementary Table 3.
† See Methods for details.

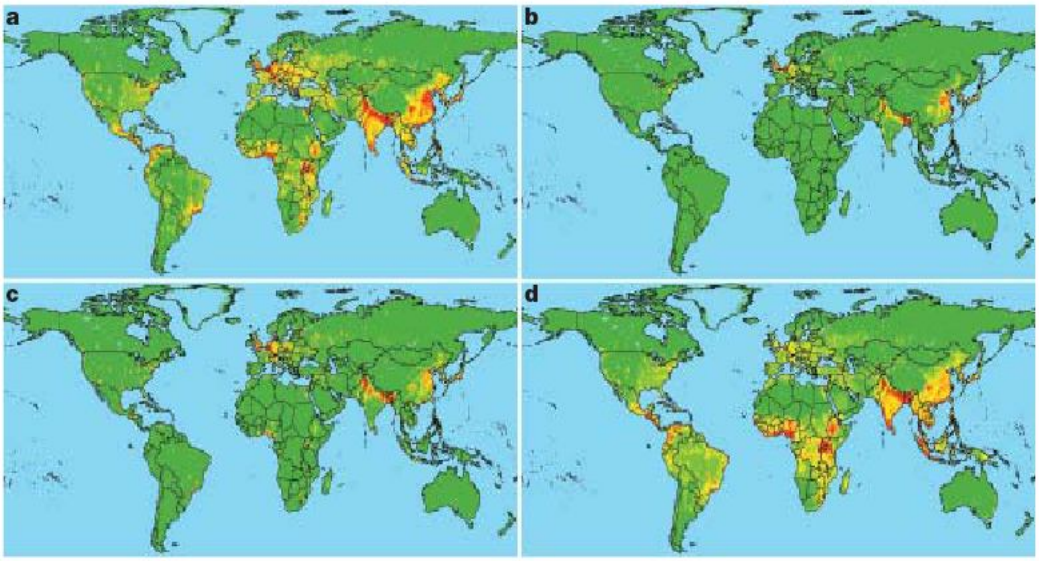

Figure 3 | Global distribution of relative risk of an EID event. Maps are derived for EID events caused by **a**, zoonotic pathogens from wildlife, **b**, zoonotic pathogens from non-wildlife, **c**, drug-resistant pathogens and **d**, vector-borne pathogens. The relative risk is calculated from regression coefficients and variable values in Table 1 (omitting the variable measuring reporting effort), categorized by standard deviations from the mean and mapped on a linear scale from green (lower values) to red (higher values).

METHODS SUMMARY

Biological, temporal and spatial data on human EID 'events' were collected from the literature from 1940 (yellow fever virus, Nuba Mountains, Sudan) until 2004 (poliovirus type 2 in Uttar Pradesh, India) ($n = 335$, see Supplementary Data for data and sources). Global allocation of scientific resources for disease surveillance has been focused on rich, developed countries (Supplementary Fig. 3). It is thus likely that EID discovery is biased both temporally (by increasing research effort into human pathogens over the period of the database) and spatially (by the uneven levels of surveillance across countries). We account for these biases by quantifying reporting effort in JID and including it in our temporal and spatial analyses. JID is the premier international journal (highest ISI impact factor 2006: http://portal.isiknowledge.com/) of human infectious disease research that publishes papers on both emerging and non-emerging infectious diseases without a specific geographical bias. To investigate the drivers of the spatial pattern of EID events, we compared the location of EID events to five socio-economic, environmental and ecological variables matched onto a terrestrial one degree grid of the globe. We carried out the spatial analyses using a multivariable logistic regression to control for co-variability between drivers, with the presence/absence of EID events as the dependent variable and all drivers plus our measure of spatial reporting bias by country as independent variables ($n = 18{,}307$ terrestrial grid cells). Analyses were conducted on subsets of the EID events—those caused by zoonotic pathogens (defined in our analyses as pathogens that originated in non-human animals) originating in wildlife and non-wildlife species, and those caused by drug-resistant and vector-borne pathogens.

Full Methods and any associated references are available in the online version of the paper at www.nature.com/nature.

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Author Contributions P.D. conceived and directed the study and co-wrote the paper with K.E.J.; K.E.J. coordinated and conducted the analyses with M.A.L., A.S., N.G.P. and D.B.; N.G.P. compiled the EID event database; and J.L.G provided the mammal distribution data. All authors were involved in the design of the study, the interpretation of the results and commented on the manuscript.

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LETTERS

Proportionally more deleterious genetic variation in European than in African populations

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Quantifying the number of deleterious mutations per diploid human genome is of crucial concern to both evolutionary and medical geneticists[1–3]. Here we combine genome-wide polymorphism data from PCR-based exon resequencing, comparative genomic data across mammalian species, and protein structure predictions to estimate the number of functionally consequential single-nucleotide polymorphisms (SNPs) carried by each of 15 African American (AA) and 20 European American (EA) individuals. We find that AAs show significantly higher levels of nucleotide heterozygosity than do EAs for all categories of functional SNPs considered, including synonymous, non-synonymous, predicted 'benign', predicted 'possibly damaging' and predicted 'probably damaging' SNPs. This result is wholly consistent with previous work showing higher overall levels of nucleotide variation in African populations than in Europeans[4]. EA individuals, in contrast, have significantly more genotypes homozygous for the derived allele at synonymous and non-synonymous SNPs and for the damaging allele at 'probably damaging' SNPs than AAs do. For SNPs segregating only in one population or the other, the proportion of non-synonymous SNPs is significantly higher in the EA sample (55.4%) than in the AA sample (47.0%; $P < 2.3 \times 10^{-37}$). We observe a similar proportional excess of SNPs that are inferred to be 'probably damaging' (15.9% in EA; 12.1% in AA; $P < 3.3 \times 10^{-11}$). Using extensive simulations, we show that this excess proportion of segregating damaging alleles in Europeans is probably a consequence of a bottleneck that Europeans experienced at about the time of the migration out of Africa.

Current estimates of the number of deleterious mutations per diploid human genome vary by several orders of magnitude. Using a correlation in inbreeding rates within consanguineous marriages and mortality, Morton *et al.*[5] estimated that each of us carries three to five lethal equivalents (that is, an allele or combination of alleles that if made homozygous would be lethal), whereas Kondrashov[6] has predicted that the number may be as high as 100 lethal equivalents. Comparative genomic methods indicate that about 38% of amino-acid-changing polymorphisms are deleterious, with 1.6 new deleterious mutations arising per individual per generation[7], whereas studies based on segregating polymorphisms estimate that each person carries between 500 and 1,200 deleterious mutations[3,8]. It is difficult to reconcile these estimates because each study used different methods and data. Furthermore, studies that used DNA sequences included data from only several hundred genes. Hence there is a crucial need for an unbiased genome-wide estimate of the number of damaging mutations carried by individuals in different populations.

We quantify the number of damaging mutations per diploid human genome by combining the Applera genome-wide survey of SNPs found by the resequencing of 20 EAs and 15 AAs[9] with comparative genomic data including the PanTro2 build of the chimpanzee genome and predictions from protein structures. After applying strict quality control criteria, the data set that we analysed contains 39,440 autosomal SNPs free of ascertainment bias, comprising 10,150 unique transcripts in the human genome (see Methods). Of these SNPs, 20,893 were synonymous (nucleotide changes that do not change the amino acid) and 18,547 were non-synonymous (nucleotide changes that change the amino acid).

At each SNP, an individual can be homozygous for the ancestral allele (carrying zero copies of the mutant allele), heterozygous (carrying one copy of the mutant allele) or homozygous for the derived allele (carrying two copies of the mutant allele). We find that an individual is heterozygous, on average, for $1{,}962.4 \pm 275.1$ (mean $\pm$ s.d.) non-synonymous SNPs (Fig. 1a and Supplementary Table 1). These numbers are an underestimate because only SNPs with high-quality sequence and a matching chimpanzee base are considered. Perhaps for these reasons, our estimate is slightly smaller than that by Cargill *et al.*[10], even after adjusting their estimate to account for the current estimated number of genes in the genome. For both synonymous and non-synonymous SNPs, AA individuals are heterozygous at a greater number of SNPs than EA individuals are (Fig. 1a; $P < 6.2 \times 10^{-10}$, Mann–Whitney *U*-test for synonymous SNPs; $P < 6.2 \times 10^{-10}$, Mann–Whitney *U*-test for non-synonymous SNPs), which is consistent with previous studies finding higher levels of genetic variability in Africa[4]. For both types of SNP we find that EA individuals are homozygous for the derived allele at a greater number of SNPs than AA individuals are (Fig. 1b; $P < 6.2 \times 10^{-10}$, Mann–Whitney *U*-test). These patterns are due largely to a greater number of SNPs fixed for the derived allele in the EA sample while segregating for two alleles in the AA sample, because when we count the number of homozygous derived genotypes per individual in the EA individuals considering only those SNPs that are polymorphic in EAs (that is, excluding SNPs that are fixed in EAs but polymorphic in AAs) and the number of homozygous derived genotypes per individual in the AA individuals considering only those SNPs polymorphic in AAs (for example excluding SNPs that are fixed in AAs and polymorphic in EAs), the EA individuals no longer have significantly more homozygous derived genotypes for both categories of SNP.

To estimate the number of damaging alleles carried by each individual in our sample, we used the PolyPhen algorithm[8,11] to predict which non-synonymous SNPs might disrupt protein function. PolyPhen predicts whether a SNP is 'benign', 'possibly damaging' or 'probably damaging' on the basis of evolutionary conservation and structural data. To assess whether 'damaging' SNPs were more likely to be deleterious, we compared the allele frequency distribution

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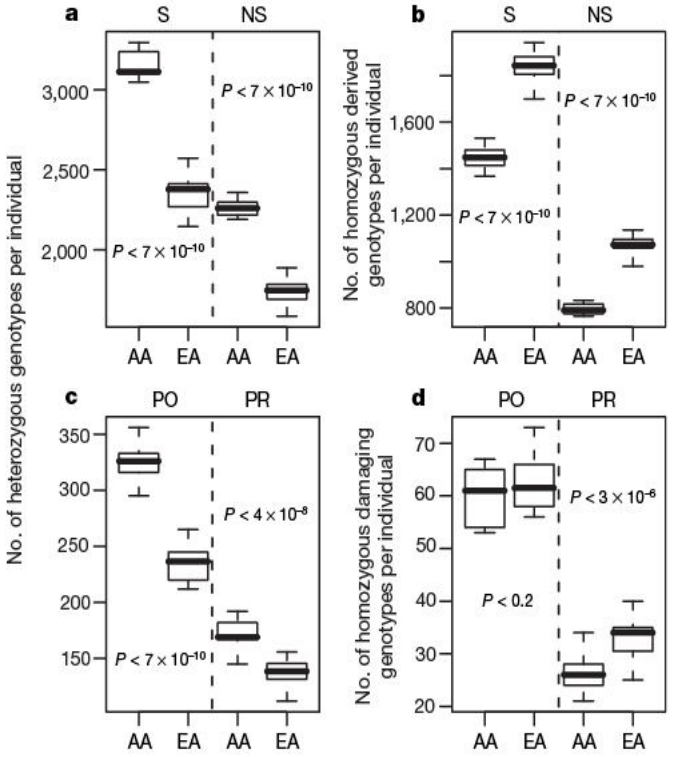

Figure 1 | Distribution of the number of heterozygous and homozygous genotypes per individual. **a**, Number of heterozygous genotypes per individual at synonymous (S) or non-synonymous (NS) SNPs. **b**, Number of genotypes homozygous for the derived allele per individual at synonymous or non-synonymous SNPs. **c**, Number of heterozygous genotypes per individual at possibly damaging (PO) or probably damaging (PR) SNPs. **d**, Number of genotypes homozygous for the damaging allele at possibly damaging or probably damaging SNPs. Dark horizontal lines within boxes indicate medians, and the whiskers indicate the ranges of the distributions.

of SNPs predicted to be 'benign', 'possibly damaging' and 'probably damaging' for each population. We find that the three distributions are significantly different from each other, with more low-frequency SNPs in the 'probably damaging' category (Table 1; $P < 5.9 \times 10^{-81}$ for AA, $P < 2.3 \times 10^{-101}$ for EA; Kruskal–Wallis test), suggesting that most SNPs classified as 'damaging' are also evolutionarily deleterious.

Figure 1c, d shows the distribution of the number of SNPs per individual where individuals were heterozygous (Fig. 1c) and homozygous for the damaging allele (Fig. 1d) for SNPs predicted to be 'possibly damaging' and 'probably damaging'. We find that an individual typically carries 426.1 damaging (here defined as 'possibly damaging' or 'probably damaging') SNPs in the heterozygous state (s.d. 65.4, range 340–534) and 91.7 in the homozygous state (s.d. 8.6, range 77–113). Because we surveyed just over 10,000 genes, the actual number of damaging mutations in a person's genome may be as much as double that given here. Every individual in our sample is heterozygous at fewer 'probably damaging' SNPs than synonymous SNPs, which is consistent with the elimination of damaging SNPs from the population by purifying selection. AAs have significantly more heterozygous genotypes than EAs for all three PolyPhen categories (Fig. 1c; $P < 6.2 \times 10^{-10}$ for 'possibly damaging' SNPs; $P < 3.7 \times 10^{-8}$ for 'probably damaging' SNPs). The two populations differ significantly in the distribution of homozygous genotypes for the damaging allele at 'probably damaging' SNPs (Fig. 1d; $P < 2.7 \times 10^{-6}$), with EAs having about 26% more homozygous damaging genotypes than AAs. The lack of a statistical difference at 'possibly damaging' SNPs ($P = 0.17$) is probably due to a lack of power because, overall, all other categories of SNPs (synonymous, non-synonymous, 'benign' and 'probably damaging') follow the same pattern of excess homozygosity for the derived/damaging allele in EAs relative to AAs.

Classical analyses of human inbreeding indicate that each individual carries 1.44–5 lethal equivalents[5,12]. However, inbreeding studies cannot determine whether a single lethal equivalent is due to one lethal allele, two alleles each with a 50% chance of lethality, ten alleles each with a 10% chance of lethality, or other combinations. Because we find that individuals carry hundreds of damaging alleles, it is likely that each lethal equivalent consists of many weakly deleterious alleles. Our finding that each person carries several hundred potentially damaging SNPs indicates that large-scale medical resequencing will be useful to find common and rare SNPs of medical consequence[2].

We next examined the distribution of synonymous and non-synonymous SNPs between AA and EA population samples (Table 1). As expected[4], there are more of both types of SNP in the AA sample than in the EA sample. However, when classifying synonymous and non-synonymous SNPs as being shared, private to AAs or private to EAs, we strongly reject homogeneity (Table 2, $P < 3.0 \times 10^{-88}$). We find the proportion of private SNPs that are non-synonymous (49.9%) to be higher than the proportion of shared SNPs that are non-synonymous (41.7%; $P < 4.3 \times 10^{-54}$), which is not surprising because non-synonymous SNPs are more likely to be at a lower frequency and thus be population specific. However, considering only the private SNPs, we find that the EA sample has a higher proportion of non-synonymous SNPs (55.4%) than the AA sample (47.0%; $P < 2.3 \times 10^{-37}$). We observed a similar significant proportional excess of private non-synonymous SNPs in an independent data set collected by the SeattleSNPs project (Supplementary Table 3 and Supplementary Notes). The SeattleSNPs data, additional quality control analyses (Supplementary Table 4 and Supplementary Notes), and a similar finding reported for the *ANGPTL4* locus[13] indicate that this pattern is not an artefact of the Applera data. Our further analyses with Yoruban samples from Nigeria collected by the International HapMap Consortium[14] support this result, indicating that it is robust to admixture (Supplementary Notes).

We propose that the proportional excess of non-synonymous polymorphism in the EA sample could be due to the varying efficacy of purifying selection resulting from differences in demographic histories between the two populations. Our hypothesis has two testable predictions: first, if this proportional excess of non-synonymous polymorphisms in EAs is due to an excess of damaging alleles, we would also expect to find a proportional increase of 'probably damaging' SNPs as predicted by PolyPhen in the EA sample; and second, we should be able to recapitulate this pattern by using simulations with reasonable demographic parameters. When dividing

Table 1 | Distribution of Applera SNPs by population and functional class

Category	Shared	Private AA	Private EA	Mean derived frequency	
				AA*	EA†
Synonymous	8,056 (58.3%)	8,958 (53.0%)	3,879 (44.6%)	0.211	0.266
Non-synonymous	5,771 (41.7%)	7,950 (47.0%)	4,826 (55.4%)	0.174	0.202
Benign	4,448 (78.6%)	5,260 (67.7%)	2,928 (62.1%)	0.200	0.238
Possibly damaging	795 (14.0%)	1,572 (20.2%)	1,035 (22.0%)	0.113	0.119
Probably damaging	422 (7.4%)	942 (12.1%)	749 (15.9%)	0.099	0.108

* Average frequency from SNPs segregating in the AA sample. No correction for ancestral misidentification was used.
† Average frequency from SNPs segregating in the EA sample. No correction for ancestral misidentification was used.

Table 2 | Results of *G*-tests of homogeneity for Table 1

Comparison	Non-synonymous versus synonymous			Benign versus possibly damaging versus probably damaging		
	G	d.f.	*P*	*G*	d.f.	*P*
Shared versus private AA versus private EA	403.1	2	3.0×10^{-88}	377.8	4	1.8×10^{-80}
Shared versus private	239.9	1	4.3×10^{-54}	329.5	2	2.9×10^{-72}
Private AA versus private EA	163.2	1	2.3×10^{-37}	48.3	2	3.3×10^{-11}

non-synonymous SNPs into the three PolyPhen categories, we find a significant excess of 'probably damaging' SNPs in private SNPs compared with shared SNPs (Tables 1 and 2). When considering only the private SNPs, we find a significantly higher proportion of 'probably damaging' SNPs in the EA sample relative to the AA sample (Tables 1 and 2; $P < 3.3 \times 10^{-11}$), supporting our hypothesis that the excess proportion of non-synonymous SNPs in the EA sample is due to a higher proportion of damaging SNPs.

To assess whether these observations are consistent with plausible demographic histories of the two populations, we developed a large-scale forward simulation program that included non-stationary demography and a negative log-normal distribution of selective effects for deleterious mutations. Our program used demographic parameters estimated from the data and the literature[15] for each population (Supplementary Table 2). For example, for the simulations in Fig. 2a, b we used a population expansion model for the AAs and a bottleneck model for the EAs (Supplementary Fig. 1). We sampled from these simulated populations and found that the proportion of non-synonymous SNPs is greater in the bottlenecked population than in a population that has expanded (Fig. 2a, Supplementary Table 2 and Supplementary Fig. 2a). Furthermore, as shown in Fig. 2a, the simulated proportions agree with the observed proportions for the Applera data set (here the proportion includes all SNPs, not just private ones). For all demographic models considered, we observed a higher proportion of non-synonymous SNPs in the population that underwent a bottleneck than in a population of constant size or in one that has expanded; however, the degree to which these other models fit the observed data is variable (Supplementary Table 2 and Supplementary Fig. 2a). For all models tested, we find that a higher proportion of SNPs in the simulated EA sample are weakly or strongly deleterious ($-0.001 < s < -0.5$) than in the simulated AA sample (Fig. 2b, Supplementary Table 2 and Supplementary Fig. 2b), which supports our hypothesis that a higher proportion of deleterious alleles have accumulated in the bottlenecked population. Our analysis illustrates that plausible models of human demography and purifying selection are sufficient to account for the observed increase in the proportion of non-synonymous SNPs in the EA sample relative to the AA sample.

To determine how the bottleneck contributed to the increased proportion of non-synonymous SNPs in the EA sample, we recorded the number of SNPs at different time points throughout our forward simulations (see Methods). Figure 2c–e shows how the number of synonymous SNPs and non-synonymous SNPs and the proportion of non-synonymous SNPs change over time for the EA and AA models described above as well as for a second bottleneck model having a shorter, but more severe, reduction in population size. At the start of the bottleneck, the proportion of non-synonymous SNPs drops below the pre-bottleneck value (because of the preferential loss of low-frequency non-synonymous SNPs). Then the proportion increases during the bottleneck as a result of the accumulation of slightly deleterious SNPs that behave almost neutrally in the small population but are eliminated efficiently from larger populations[16]. Once the population expands, the proportion of non-synonymous SNPs increases markedly because the increase in population size results in many more mutations (most of which are non-synonymous, because of the genetic code) entering the population (Fig. 2c, d). Because growth was recent, purifying selection has not had sufficient time to decrease the proportion of non-synonymous SNPs to the equilibrium value for the larger population. A related effect has been noted in spatial expansion models, in which deleterious mutations can 'surf' to high frequency on the edge of the expansion[17]. Our simulations for African demography suggest that once the African population expanded, the proportion of non-synonymous SNPs also increased initially. However, because

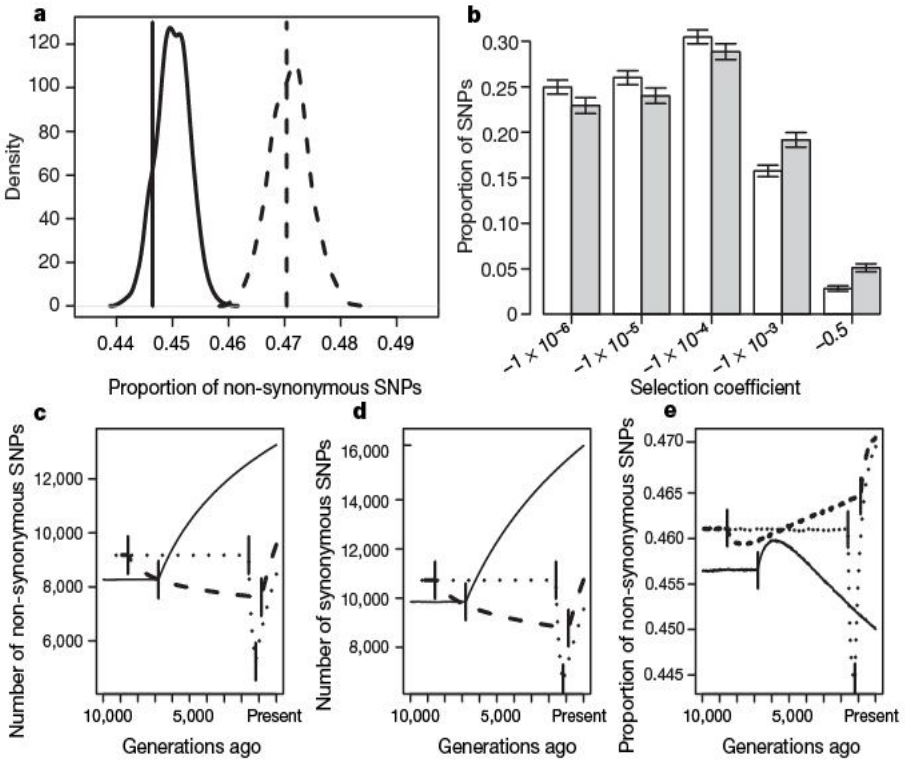

Figure 2 | Demography and selection can cause a proportional excess of non-synonymous SNPs in Europeans. **a**, **b**, Results of forward simulations of a population that expanded (AA 2 in Supplementary Table 2), to represent the AA population and a population that experienced a bottleneck to represent the EA population (EA 1 in Supplementary Table 2). **a**, Distribution of the proportion of non-synonymous SNPs segregating in samples simulated under European (dashed curve) and African (solid curve) demographic models. Vertical lines show the observed proportions in the Applera data set. **b**, Distribution of selection coefficients for simulated SNPs in the AA (white bars) and the EA (grey bars) samples. The labels on the *x* axis are the more negative limits of the bins. Error bars denote 95% intervals on the proportion of SNPs in each group. **c**–**e**, Expected distribution of SNPs over time during a population expansion (AA 2, solid lines), a long, mild bottleneck (EA 1, dashed lines) and a short, severe bottleneck (EA 6, dotted lines). Time moves forward from left to right. Solid vertical lines indicate when the populations changed size. Further details are given in Supplementary Table 2. **c**, The number of non-synonymous SNPs. **d**, The number of synonymous SNPs. **e**, The proportion of non-synonymous SNPs.

the African expansion occurred farther back in time than the most recent European expansion, the proportion of non-synonymous SNPs has had more time to decrease closer to the equilibrium value in the AA sample. At the present time, the absolute numbers of SNPs are higher in the non-bottleneck model (AA 2) than in the bottleneck models (EA 1 and EA 6). The bottleneck dynamics were robust to the distribution of selective effects used in our simulations (Supplementary Fig. 3).

Thus, both the PolyPhen analysis and the forward simulations suggest that, given the lower levels of genetic diversity found in Europeans than in Africans, the former have a higher proportion of deleterious alleles, which can be explained by the 'out of Africa' bottleneck and subsequent expansion that outbred European populations endured. This result is important for two reasons. First, whereas previous work has highlighted examples of European-specific positive selection[14,18–21], the importance of adaptations for the evolution of European populations needs to be tempered by our finding that negative selection is less effective at removing slightly deleterious alleles from European populations. Second, the idea that bottlenecks and founder effects could lead to an increase in damaging alleles in human populations was historically reserved for isolated populations that experienced severe founder effects (for example Ashkenazi Jews[22] and Finns[23]). Our work suggests that the interaction of demographic processes and purifying selection can have an important impact on the distribution of deleterious variation, even in populations that did not undergo a severe founder effect.

METHODS SUMMARY

We used an improved bioinformatics pipeline to analyse SNPs, described in ref. 9. We mapped the SNPs to the RefSeq v18 gene model to determine whether they were synonymous or non-synonymous. Ancestral and derived states for each SNP were determined with the syntenic net alignments between hg18 and panTro2 (refs 24, 25). When counting the number of genotypes per individual, we added a correction for misidentification of the ancestral allele[26]. SNPs were dropped from the analysis if they failed to meet our bioinformatics quality controls, but we did not filter SNPs on the basis of frequency.

To predict whether a non-synonymous SNP will damage protein function, we used an updated version of PolyPhen that has false-positive and false-negative rates below about 15% (Supplementary Methods). When counting the number of damaging genotypes per individual, we used the subset of SNPs in which the predicted damaging allele was the derived allele.

An additional four AA individuals were sequenced, but we did not include them (or SNPs private to them) in further analyses because we determined that they had substantially more European admixture than the other AAs (Supplementary Methods, Supplementary Table 5 and Supplementary Fig. 4). If our estimates of admixture are not perfect, this should not drastically affect the comparisons of different classes of SNPs, making our analysis robust to this problem (Supplementary Notes). The Coriell sample numbers for the individuals used in our study are given in Supplementary Table 1.

To test whether the higher proportion of non-synonymous SNPs in EAs than in AAs could be due to the different demographic histories of the two populations, we used forward simulations that allowed us to model demography and purifying selection. We considered a range of demographic models for both populations (Supplementary Table 2) and a distribution of selective effects for non-synonymous SNPs.

Full Methods and any associated references are available in the online version of the paper at www.nature.com/nature.

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Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

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Author Contributions K.E.L. and C.D.B. conceived of the original design of the project. J.J.S. and T.J.W. directed the collection of the sequence data by Celera Genomics. K.E.L., A.R.I., A.R.B., R.D.H., S.S. and M.J.H. designed the bioinformatics pipeline and analysed the data with direction from S.S., R.N., A.G.C. and C.D.B. K.E.L. performed the simulations. K.E.L., A.G.C. and C.D.B. wrote the paper with input from all authors.

Author Information Accession numbers for the SNP markers analysed in this study are dbSNP numbers ss48401226–ss48429818 and ss48429821–ss48431291, submitted under the handle APPLERA_GI, and ss86236910–ss86273113, submitted under the handle CORNELL. Reprints and permissions information is available at www.nature.com/reprints. Correspondence and requests for materials should be addressed to C.D.B. (cdb28@cornell.edu).

LETTERS

Genotype, haplotype and copy-number variation in worldwide human populations

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Genome-wide patterns of variation across individuals provide a powerful source of data for uncovering the history of migration, range expansion, and adaptation of the human species. However, high-resolution surveys of variation in genotype, haplotype and copy number have generally focused on a small number of population groups[1–3]. Here we report the analysis of high-quality genotypes at 525,910 single-nucleotide polymorphisms (SNPs) and 396 copy-number-variable loci in a worldwide sample of 29 populations. Analysis of SNP genotypes yields strongly supported fine-scale inferences about population structure. Increasing linkage disequilibrium is observed with increasing geographic distance from Africa, as expected under a serial founder effect for the out-of-Africa spread of human populations. New approaches for haplotype analysis produce inferences about population structure that complement results based on unphased SNPs. Despite a difference from SNPs in the frequency spectrum of the copy-number variants (CNVs) detected—including a comparatively large number of CNVs in previously unexamined populations from Oceania and the Americas—the global distribution of CNVs largely accords with population structure analyses for SNP data sets of similar size. Our results produce new inferences about inter-population variation, support the utility of CNVs in human population-genetic research, and serve as a genomic resource for human-genetic studies in diverse worldwide populations.

The Human Genome Diversity Project (HGDP) was initiated for the purpose of assessing worldwide genetic diversity, providing cell lines maintained at the Centre d'Étude du Polymorphisme Humain (CEPH) for use in population-genetic studies[4]. We genotyped a geographically broad subset of 485 individuals from the HGDP–CEPH panel, with complete inclusion of HGDP–CEPH Africans (Supplementary Fig. 1). After correction for sample size differences across geographic regions[5], 81.17% of SNP alleles were observed in all five of the main regions (Fig. 1a). The next most frequently observed geographic distributions represented alleles found everywhere except Oceania (3.80%), everywhere except the Americas (3.01%), and everywhere except Africa (2.20%). Regionally private alleles were uncommon: 0.91% for Africa, 0.75% for Eurasia (Europe, Central/South Asia and the Middle East, including North Africa), and near zero for other regions.

Genomic analysis of population structure produced higher-resolution inferences than have previously been obtained. In a neighbour-joining population tree based on allele-sharing distance, with one exception, all internal branches were supported by all 1,000 bootstrap replicates across loci (Fig. 1b); nine replicates grouped the Adygei population with Russians and Basques. The tree supports the clustering of each of the main geographic regions and contains a separation of African hunter-gatherers (San, Mbuti and Biaka) from other Africans.

Bayesian cluster analysis[6] was largely concordant with previous analyses of microsatellite and short insertion–deletion polymorphisms[7–9]. Analysis with six clusters revealed groupings corresponding to five geographic subdivisions separated by major barriers, with a cline longitudinally across Asia and with a sixth cluster centred on the Kalash population of Pakistan (Fig. 1c). Within geographic regions, the cluster analysis subdivided groupings that were observed previously with fewer markers[9] (Fig. 1c and Supplementary Fig. 2).

Multidimensional scaling (MDS) separated the populations of different geographic regions (Fig. 1d), including Europe, Central/South Asia and the Middle East, which clustered together in the global bayesian analysis. Within regions, MDS split the individuals of distinct populations into distinct clusters (Supplementary Fig. 3), even in some cases for which bayesian analysis produced little separation between populations. The possibility of placing the MDS graph in approximate geographical orientation, with latitude and longitude representing the vertical and horizontal axes, suggests that geographic distance is a primary determinant of human genetic differentiation[10,11]. This view is supported by a linear increase in genetic distance with geographic distance from East Africa (Fig. 2a).

Linkage disequilibrium (LD), as obtained with the homozygosity-based HR^2 measure[12], declined as a function of physical distance, with the highest values occurring in the Americas, followed by Oceania, East Asia, Eurasia and Africa (Fig. 2b). Only two populations deviated from this pattern—Maya, a potentially admixed group, and Kalash, a population isolate. Although reduced LD has consistently been

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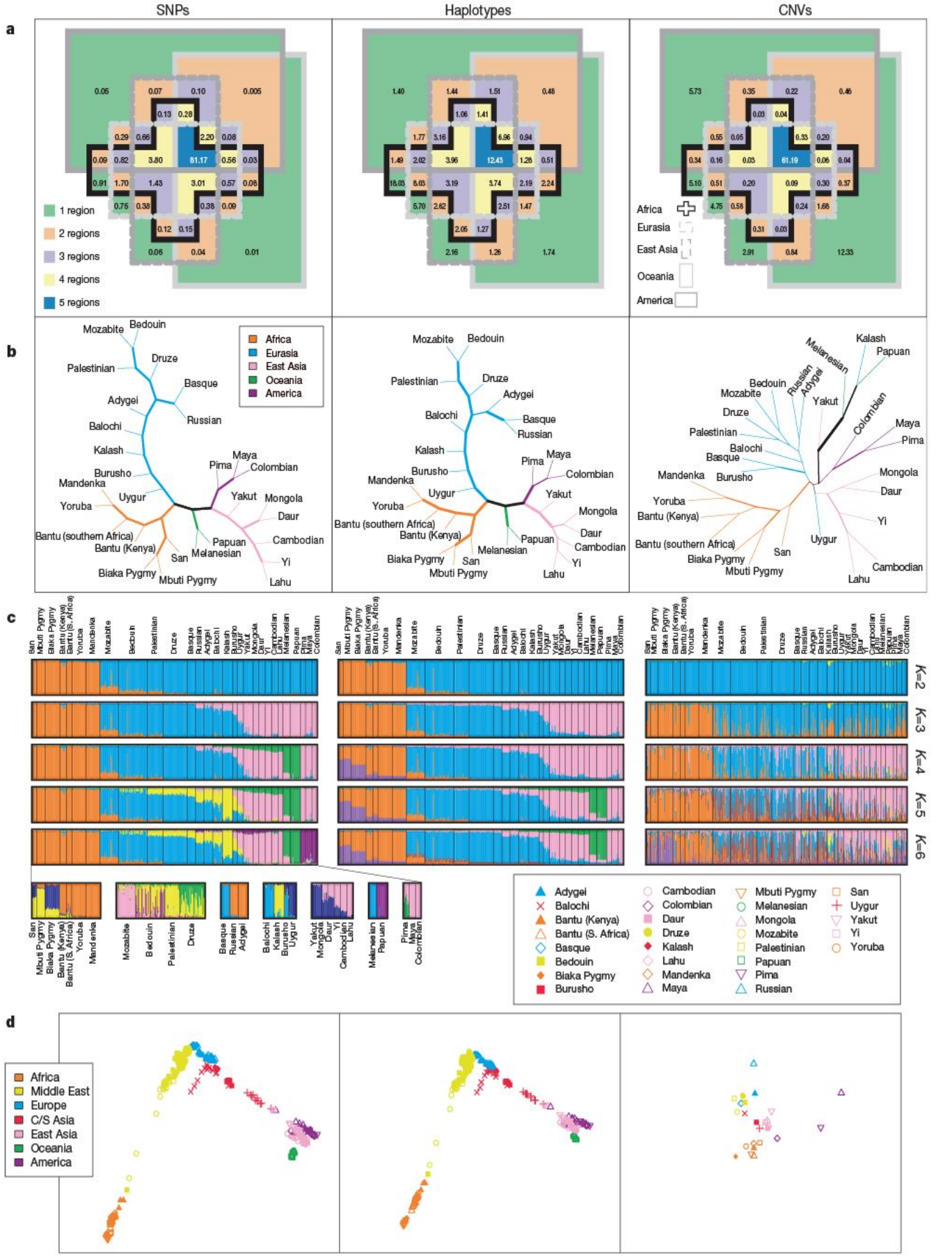

Figure 1 | SNP, haplotype, and copy-number variation across populations. **a**, Venn diagram of the percentages of alleles with particular geographic distributions. **b**, Neighbour-joining trees of population relationships. Internal branch lengths are proportional to bootstrap support. Lines of intermediate thickness represent internal branches with more than 50% bootstrap support, and the thickest lines represent more than 95% support. **c**, Population structure inferred by bayesian clustering. Each individual is shown as a thin vertical line partitioned into K coloured components representing inferred membership in K genetic clusters. The bottom row provides inferred population structure for each geographic region. **d**, MDS representations of genetic distances between individuals (SNPs and haplotypes) and populations (CNVs). C/S Asia, Central/South Asia.

observed in Africa, LD levels in non-African groups have been difficult to rank[13–16]. We observed that, with high precision, LD increased with geographic distance from East Africa (Fig. 2c). This pattern matches the prediction from a model of sequential founder effects during spatial expansion from Africa[11], because such founder effects would be expected to increase LD at each step of the expansion[15,17].

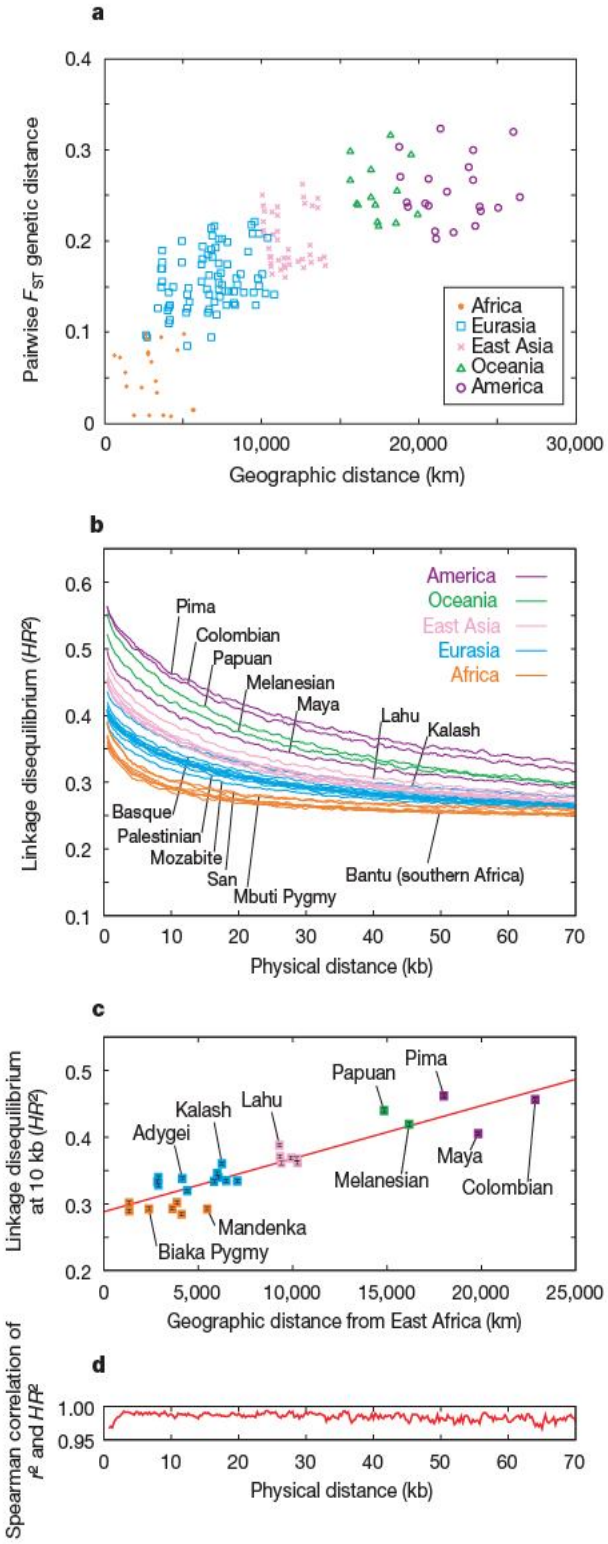

Figure 2 | Genetic distance and linkage disequilibrium. **a**, F_{ST} genetic distance as a function of land-based geographic distance from East Africa. **b**, LD as a function of physical distance. kb, kilobases. **c**, LD as a function of geographic distance from East Africa. Error bars (smaller than symbol size) represent the mean ± 1.96 times the s.e.m. **d**, Correlation of population rank orders by LD, comparing HR^2 applied to unphased data and r^2 applied to phased data. LD calculations are adjusted for sample size differences across populations by sampling five random individuals (HR^2) or ten random haplotypes (r^2) per population at each SNP pair.

To circumvent possible biases in SNP selection procedures[13], we also analysed estimated haplotypes. In comparison with the pattern for HR^2, a nearly identical LD decay was observed with the r^2 measure applied to phased data (Supplementary Fig. 4). The correlation of population ranks by HR^2 and r^2 levels exceeded 0.95 across a wide range of physical distances (Fig. 2d).

For further assessment of haplotype variation, we devised a new approach that avoided the difficulty of choosing window lengths for haplotypic analysis. Variation is summarized locally at each point in the genome by using a collection of 20 'haplotype clusters', each of which represents a group of haplotypes that overlap the point. For every population, frequencies for the various haplotype clusters are estimated at each SNP. Example illustrations of these frequencies are shown in Fig. 3 in the vicinity of the lactase gene (*LCT*). A decrease in haplotype diversity in Europe, particularly in the CEU population (Utah residents with ancestry from northern and western Europe), is apparent from the predominance of a single haplotype cluster well beyond *LCT*. This pattern accords with evidence that *LCT* has recently undergone a selective sweep[1,18,19], because such sweeps are expected to generate high-frequency uninterrupted haplotypes surrounding the selected region. By contrast, the reduced diversity in the Americas and Oceania probably reflects founder events and consequently greater haplotype lengths genome-wide (Supplementary Figs 5–7).

To make use of haplotypes in population structure analysis, we generated ten haplotype cluster data sets, each of which assigned each individual two haplotype clusters at every point along the genome, with both cluster memberships ranging from 1 to 20. The ten data sets were then analysed with the same methods as those used for unphased genotypes, treating distinct clusters in the same manner as distinct alleles.

Only 12.43% of haplotype clusters were observed in all five regions, whereas 18.03% were private to Africa (Fig. 1a). Geographically localized haplotype clusters were considerably more common than localized SNP alleles, with 51.87% of clusters being found in at most two regions, in contrast with 4.66% of SNP alleles. Despite these differences in geographic distributions, the haplotype-based neighbour-joining tree had an identical shape to the SNP-based tree, except for a Basque–Russian–Adygei grouping (Fig. 1b), and haplotype-based and SNP-based MDS plots were extremely similar (Fig. 1d). Bayesian clusters with haplotype data matched those in the unphased analysis, except that the haplotypically diverse Africans quickly split into a cluster partly corresponding to African hunter-gatherers and a cluster for the other African populations, and Native Americans and Kalash did not separate (Fig. 1c). The general agreement of SNP-based and haplotype-based analyses suggests that at the high density considered, unphased SNPs provide considerable population structure information, although haplotype data can contribute an additional informative component for population structure analysis. Haplotype-based subdivision of Africans suggests a preference for splitting the highest-diversity groups over separating relatively isolated populations—Kalash and Native Americans—whose haplotypes largely represent subsets of those seen in neighbouring groups.

In conjunction with SNP typing, we identified CNVs by using PennCNV[20], a CNV-calling program that relies on SNP allele frequencies, SNP spacing, and genotyping signal intensities and allelic intensity ratios normalized by signals for a reference panel. We detected 3,552 CNVs at 1,428 copy-number-variable loci, including 507 loci at which CNVs have not previously been reported. Sufficient reliability of CNV genotypes for population-genetic analysis is supported by the observation that all CNVs detectable by using consecutive heterozygous genotypes on male X chromosomes were also identified from signal intensity (Supplementary Figs 8 and 9), by a combined false-positive and false-negative rate of 9% reported for PennCNV[20], and by a false-positive rate below 0.7% as estimated from duplicate samples[21] (Supplementary Figs 10 and 11). For analyses of population structure (Fig. 1), the CNV data set

was restricted to 396 non-singleton autosomal loci in 405 unrelated individuals.

CNVs tended to have low frequencies worldwide: only one CNV frequency exceeded 10% (Supplementary Fig. 12). Within geographic regions, however, higher-frequency CNVs were more common, especially in Oceania and the Americas (Fig. 4a and Supplementary Fig. 13). Consistent with this trend, three of the four populations with the greatest numbers of CNVs detected per individual occurred in these regions, the fourth being Kalash (Fig. 4b). In contrast with their usual reduced variation[11,13], populations from Oceania and the Americas had more CNV loci and more previously unobserved CNV loci than most other populations. The number of private CNVs was larger for Oceania than for Africa and Eurasia (Fig. 1a), a pattern not observed with SNP and haplotype variation. Private CNVs were more common than private SNP alleles, and for CNVs the percentage observed in all five regions, 61.19%, was smaller than for SNPs. The excess of rare and localized variants is probably due in part to comparison with preselected known SNPs, but it accords with a skew towards rare variants in CNVs observed with other genotyping technologies[22,23]. However, some bias may exist in CNV detection; as a result of difficulties in detecting high-frequency CNVs from comparisons against reference intensities[24], the absence from the reference panel of Kalash and populations from Oceania and the Americas may have increased the potential for identifying CNVs in these groups. In such distinctive populations, unusual intensity signals for deletions or duplications are less likely to have been diluted by inclusion in the reference panel of individuals with an atypical copy number.

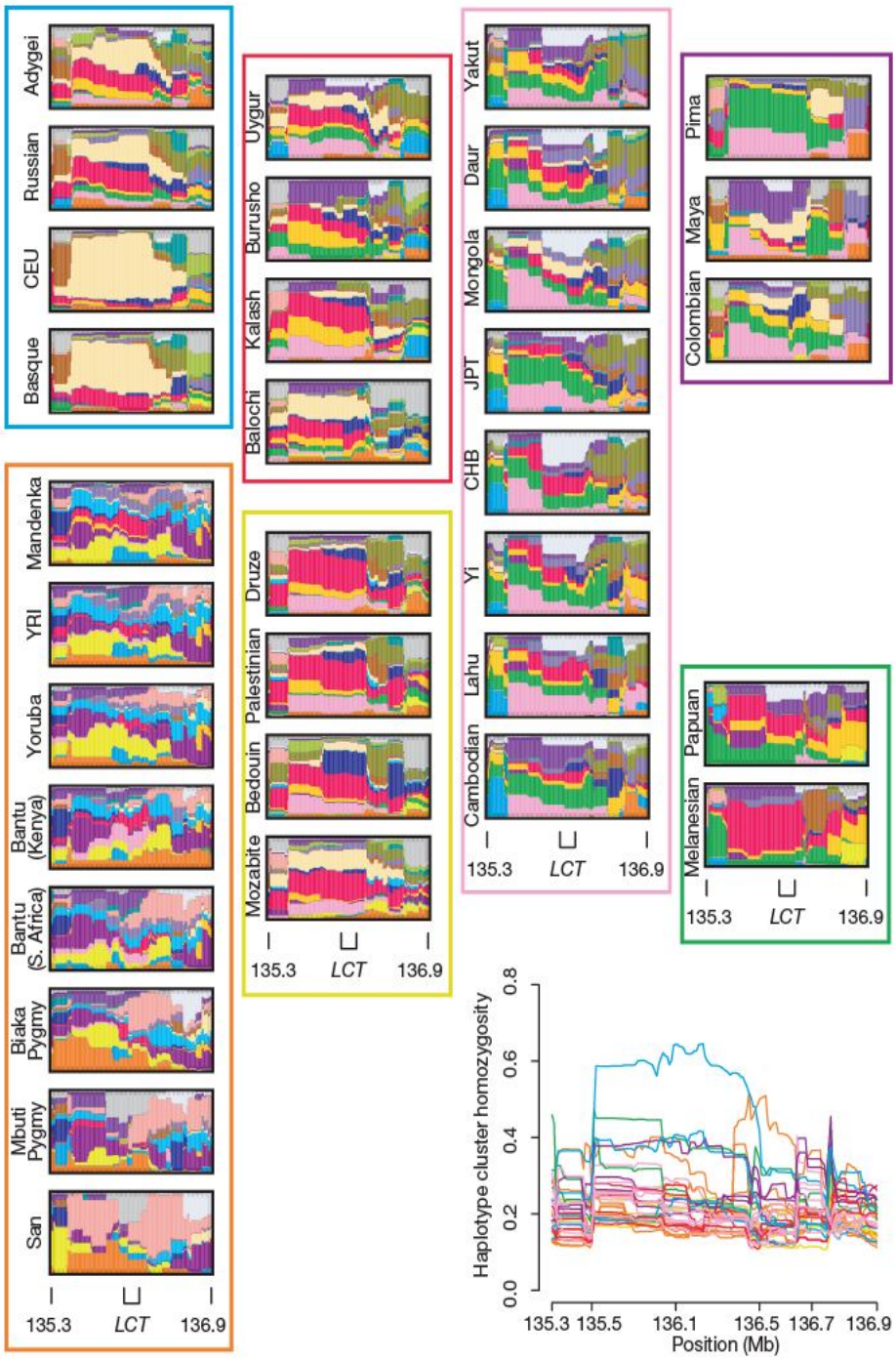

Figure 3 | Haplotype cluster frequencies for 156 consecutive SNPs on chromosome 2 in the region surrounding the *LCT* gene (136.373–136.478 megabases). At each SNP, relative frequencies of haplotype clusters are displayed on a thin vertical line. Each colour depicts a haplotype cluster, and the proportion in a colour gives the frequency of 1 of 20 distinct clusters. Interpretation of colours is made locally, as clustering varies along the chromosome, reflecting a gradual decay of LD. Moving horizontally, changes in colour patterns illustrate the change in haplotypic composition across physical position. CEU, Utah residents with ancestry from northern and western Europe; CHB, Han Chinese from Beijing; JPT, Japanese from Tokyo; YRI, Yoruba from Ibadan, Nigeria.

Partial similarity was observed between population structure inferred for CNVs and that inferred from considerably larger SNP and haplotype data sets. In the population tree, major geographic regions largely formed separate branches, but with different lower-level groupings than in the SNP and haplotype trees, and with less support (Fig. 1b); the unexpected grouping of Kalash, Melanesian and Papuan probably results from long-branch attraction during neighbour-joining analysis of their large numbers of CNVs (Supplementary Tables 1 and 2). Bayesian cluster analysis separated populations from Africa, Eurasia and the combination of East Asia, Oceania and the Americas, but with considerable variation across individuals (Fig. 1c). MDS revealed some degree of geographic clustering, but only after removal of the three outliers that also appear in the population tree (Fig. 1d and Supplementary Fig. 14). The degree of difference between CNV and SNP population structure results is comparable to that obtained with subsets of the SNP data set with the same size as the CNV data set (Supplementary Figs 15 and 16, and Supplementary Tables 3 and 4). Thus, partial correspondence of CNV population structure patterns to those observed for SNPs and haplotypes supports the general reliability of the CNV genotyping and suggests some similarity in the evolutionary history of CNV loci to the histories of other types of marker.

The availability of worldwide high-density SNP data will be important for improving the prospects for disease-gene mapping in a broad set of populations. By employing methods that make use of high-resolution data sets to impute genotypes in study samples[25], it will be possible to increase power to detect associations in diverse populations for which such data have not previously been available. The data also provide the basis for refining informative marker sets in contexts such as multi-population SNP tagging[26], admixture mapping and ancestry inference, and for evaluating SNP tagging of CNVs for disease association tests[3,22]. Because effective tagging may require high r^2 values between markers, and because high r^2 occurs only for markers with similar allele frequencies[27], a difference in SNP and CNV allele frequency spectra suggests that ideal SNP sets for tagging CNVs may require a considerable fraction of rare variants. Finally, our detection of novel copy-number-variable loci in a population panel broader than those used in previous CNV analyses highlights the importance of considering diverse worldwide populations for full characterization of the pattern of human genetic variation.

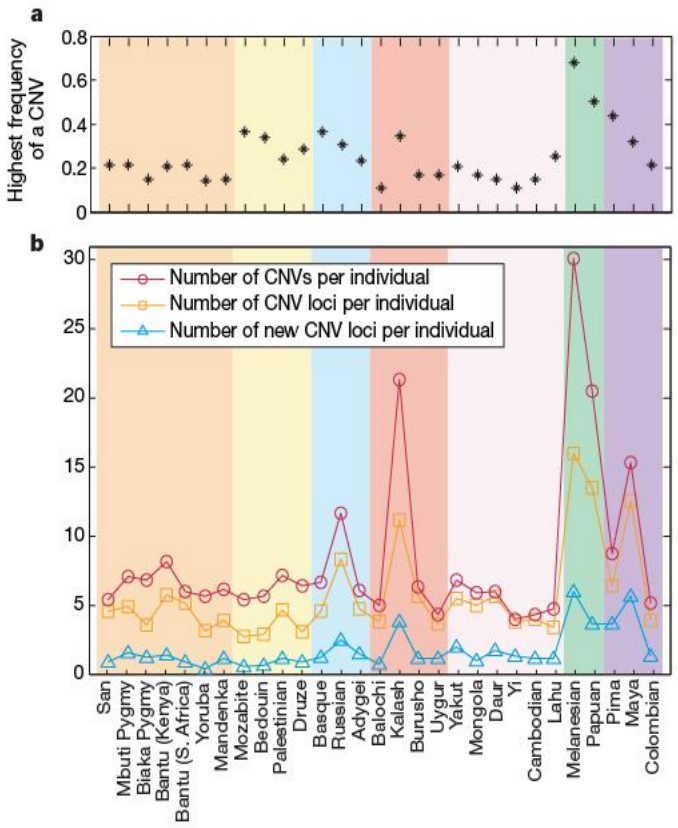

Figure 4 | CNVs across populations, based on 3,552 CNVs at 1,428 copy-number-variable loci. a, Highest frequency of any autosomal CNV in each of 29 populations. **b**, Mean number of CNVs observed per individual. Number of CNVs per individual refers to the number of CNVs considering all individuals in a population, divided by sample size; number of (new) CNV loci refers to the number of (new) CNV loci polymorphic in a population, divided by sample size. To be identified as new, we required that a CNV not overlap with existing CNVs in the Database of Genomic Variants[30] (version hg18.v3). Background colours indicate geographic regions.

METHODS SUMMARY

SNPs. Genotyping used Illumina Infinium HumanHap550 BeadChips. HGDP–CEPH genotypes were augmented with HumanHap550 genotypes of 112 HapMap individuals. Most analyses used 512,762 high-quality autosomal SNPs in 443 unrelated HGDP–CEPH individuals. Data appear at http://neurogenetics.nia.nih.gov/paperdata/public/ and http://www.cephb.fr/hgdp-cephdb/.

Haplotypes. Phasing with fastPHASE[28] used 20 haplotype clusters, combining HGDP–CEPH and HapMap individuals, and employing geographic region labels to enhance accuracy[13]. Relatives were subsequently removed. For each individual, at each SNP, probabilities were obtained for the haplotype cluster memberships of the two unobserved haplotypes of the individual, averaging across individuals to produce cluster 'frequencies' for each population. Haplotype cluster data sets were constructed by taking (for each chromosome) ten independent samples from the conditional distribution of chromosome-wide memberships given the unphased genotypes and the estimated parameters of the model underlying fastPHASE. Cluster data set preparation for population structure analysis ignored geographic labels.

CNVs. CNV detection employed a ten-SNP minimum to increase the reliability of calls[20]. Copy-number-variable loci were identified as regions with CNVs. One-copy changes (one allele duplicated or deleted) were tabulated as one CNV; two-copy changes were tabulated as two CNVs.

Data analysis. Rarefaction computations[5] of mean numbers of variants per locus private to each of 31 combinations of geographic regions used equal samples of 35 chromosomes per region. Percentages shown equal these 31 values, normalized by their sum. Trees were obtained from 1,000 bootstraps across loci; for haplotypes, bootstraps were split evenly across the ten data sets. Bayesian clustering used 40 replicates, using 1% of the SNP and haplotype data to avoid markers in LD. 'Replicates' included different 1% subsets (SNPs, haplotypes), different data sets (haplotypes) and separate runs with identical data (SNPs, haplotypes, CNVs). CLUMPP[29] was used to identify shared modes. For SNPs and CNVs, MDS used allele-sharing distance between individuals; for haplotypes, it used euclidean distance between cluster membership vectors.

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Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

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Author Contributions N.A.R. and A.B.S. wish to be regarded as joint last authors.

Author Information The array data described in this paper are deposited in the Gene Expression Omnibus (www.ncbi.nlm.nih.gov/geo) under accession number GSE10331. Reprints and permissions information is available at www.nature.com/reprints. Correspondence and requests for materials should be addressed to N.A.R. (rnoah@umich.edu) or A.B.S. (singleta@mail.nih.gov).

LETTERS

A role for adult TLX-positive neural stem cells in learning and behaviour

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Neurogenesis persists in the adult brain and can be regulated by a plethora of external stimuli, such as learning, memory, exercise, environment and stress[1]. Although newly generated neurons are able to migrate and preferentially incorporate into the neural network[2–5], how these cells are molecularly regulated and whether they are required for any normal brain function are unresolved questions[6]. The adult neural stem cell pool is composed of orphan nuclear receptor TLX-positive cells[7]. Here, using genetic approaches in mice, we demonstrate that TLX (also called NR2E1) regulates adult neural stem cell proliferation in a cell-autonomous manner by controlling a defined genetic network implicated in cell proliferation and growth. Consequently, specific removal of TLX from the adult mouse brain through inducible recombination results in a significant reduction of stem cell proliferation and a marked decrement in spatial learning. In contrast, the resulting suppression of adult neurogenesis does not affect contextual fear conditioning, locomotion or diurnal rhythmic activities, indicating a more selective contribution of newly generated neurons to specific cognitive functions.

Global deletion of TLX during development leads to microencephaly, retinal dystrophy, blindness and aggression[8,9]. To facilitate an analysis of the function of TLX in adult neural stem cells (NSCs), we created a conditional allele by flanking exon 2 with two *loxP* sites. The resulting mouse strain is hereafter referred to as $Tlx^{f/f}$ (Supplementary Fig. 1).

Using β-gal-based fluorescence-activated cell sorting[7], we isolated *Tlx*-expressing NSCs from adult $Tlx^{f/Z}$ mice, in which one allele of *Tlx* is replaced with the *lacZ* marker and the other allele is flanked by *loxP* sites but is still functional. These purified adult cells have the ability to self-renew and differentiate to all three neural lineages[7]. Infection with a Cre-expressing virus results in specific deletion of the second *Tlx* allele and leads to a >80% reduction in dividing cells after 36 h of infection (Fig. 1a, b), suggesting that TLX is essential for adult NSC proliferation *in vitro*.

We then tested whether TLX is required cell autonomously. Low densities of isolated NSCs were infected with Cre-expressing virus for 10 h, after which uninfected virus was removed and twofold more uninfected wild-type cells were added. After 36 h in growth media, proliferation of infected cells (green fluorescent protein (GFP)-positive) was scored by staining for phosphorylated histone H3 (p-H3), a marker for mitosis. We found that deletion of *Tlx* in infected cells resulted in a more than 80% reduction in proliferation (Fig. 1c, d), indicating that growth factors and the presence of surrounding wild-type cells cannot rescue the loss of TLX function in the infected cells and suggesting that TLX is required cell autonomously for NSC proliferation.

To identify TLX-dependent molecular targets, we isolated two populations of *Tlx*-expressing NSCs from adult male brains. One population, designated $Tlx^{f/z;CreER}$ NSC, harbours a floxed allele of *Tlx* and a constitutively expressed transgene, $CreER^{TM}$, which encodes a fusion of Cre recombinase and a modified, tamoxifen (TM)-responsive ligand-binding domain of oestrogen receptor[10]. Addition of tamoxifen to the culture medium leads to a temporally controlled robust deletion of the floxed allele of *Tlx* (Fig. 2a). The control population ($Tlx^{f/z}$ NSC) does not contain the $CreER^{TM}$ transgene; thus, treatment with tamoxifen has no effect on *Tlx* mRNA (Fig. 2a).

Using total RNA isolated from these two populations of cells after 36 h or 60 h of treatment with tamoxifen or vehicle, we analysed all genes whose expression was altered by at least 1.39-fold. After exclusion of tamoxifen-induced changes in control cells (36 and 99 genes at 36 h and 60 h, respectively), the number of genes with altered expression in response to deletion of *Tlx* was found to be 432 genes at 36 h and 607 genes at 60 h (Fig. 2b). Among these genes, 53.9% and 51.7% are upregulated at 36 h and 60 h, respectively (Supplementary data 1–3). Further analysis revealed that 206 genes had altered expression levels at both 36 h and 60 h after tamoxifen-induced

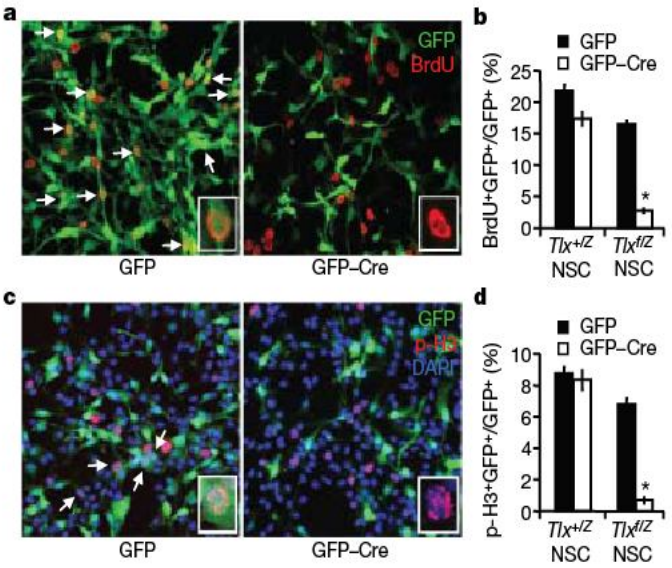

Figure 1 | Cell-autonomous requirement of TLX for adult NSC proliferation. **a**, $Tlx^{f/Z}$ NSCs were stained for GFP and BrdU after infection with adenovirus expressing GFP alone or GFP–Cre. Arrows, GFP^+BrdU^+ cells. Insets are stained cells at a higher magnification. **b**, Percentage of proliferating NSCs among infected cells. $Tlx^{+/Z}$ NSCs with one intact wild-type allele were used as controls. **c**, $Tlx^{f/Z}$ NSCs stained for GFP, mitotic marker phospho-H3 (p-H3) and the nucleus (DAPI) after virus infection. Arrows, GFP and p-H3 co-labelled cells. Insets are stained cells at a higher magnification. **d**, Percentage of infected cells undergoing mitosis. Mean ± s.e.m.; $n = 4$; asterisk, $P < 0.001$.

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deletion of *Tlx*. Of these, 21.8% are involved in regulation of cell cycle, proliferation, or DNA replication (Fig. 2b), some of which were further confirmed by quantitative RT–PCR on independent samples (Fig. 2c).

Because *Tlx* expression is restricted to the forebrain, we could use the inducible *CreER*^{TM} system to test whether it is required for adult neurogenesis *in vivo*[9–12]. Using adult reporter mice treated for 8 days with tamoxifen, we observed high recombination efficiencies in the hippocampus and ventricular regions and in proliferating NSCs (Supplementary Fig. 2a, c). Moreover, there was about an 80% reduction of *Tlx* mRNA in the forebrain and olfactory bulbs but not in the retina (Supplementary Fig. 2b), preserving visual competency (see below).

We treated 8-week-old mice with vehicle or tamoxifen for 8 days and injected 5-bromodeoxyuridine (BrdU) at various time points to label dividing cells. Although careful examination of the body weight, brain weight or brain morphology did not reveal observable changes in any of the treated mice (Supplementary Fig. 3), inducible deletion of *Tlx* resulted in a marked reduction of BrdU-labelled cells in the dentate gyrus when examined at 4 weeks or at 5.5 months (66.2% and 67.6% reduction compared to tamoxifen-treated controls, respectively; Fig. 3a–c). Additional controls were used to exclude the possibility of a Cre-mediated toxic effect[13] (Supplementary Fig. 4).

To test whether the remaining cells still responded to voluntary running with enhanced proliferation[14], we subjected the mice to 3 weeks of running 1 week after treatment and analysed the proliferation of NSCs by BrdU incorporation (Fig. 3d). Despite a marked reduction of BrdU-positive cells, the total number of dividing cells in the exercised *Tlx*-deleted brains was increased (~2-fold) compared to non-runners (compare Fig. 3d to 3b), indicating that voluntary exercise is still able to stimulate the proliferation of the remaining NSCs but cannot fully compensate for those cells lost owing to *Tlx* deletion.

Long-term effects of *Tlx* deletion on NSC survival and adult neurogenesis in the dentate gyrus were analysed 4 weeks after the last injection of BrdU (Fig. 3e–g). We found that the total number of BrdU-positive cells was markedly reduced in *Tlx*-deficient mice (Fig. 3e, 77% decrease compared to tamoxifen-treated controls). In contrast, the survival rate, which was measured as the ratio of BrdU-positive cells 4 weeks after labelling to that at 1 day after BrdU incorporation, was not significantly different from controls, indicating that deletion of *Tlx* has a minimal effect on the residual wild-type NSCs (Fig. 3f). Using NeuN as a mature neuronal marker, we observed a more than 80% reduction of NeuN and BrdU double-labelled cells (Fig. 3g). Together, these data suggest that TLX is essential for maintaining the NSC pool and the associated neurogenesis in adult brains.

Recent data indicate that adult neurogenesis is essential for contextual fear conditioning[15,16]. To test this possibility, we evaluated a cohort of mice 4 weeks after tamoxifen or vehicle treatment in the contextual fear conditioning paradigm. Unexpectedly, the learning ability of neurogenesis-deficient mice (*Tlx*$^{f/f;CreER}$ plus tamoxifen)

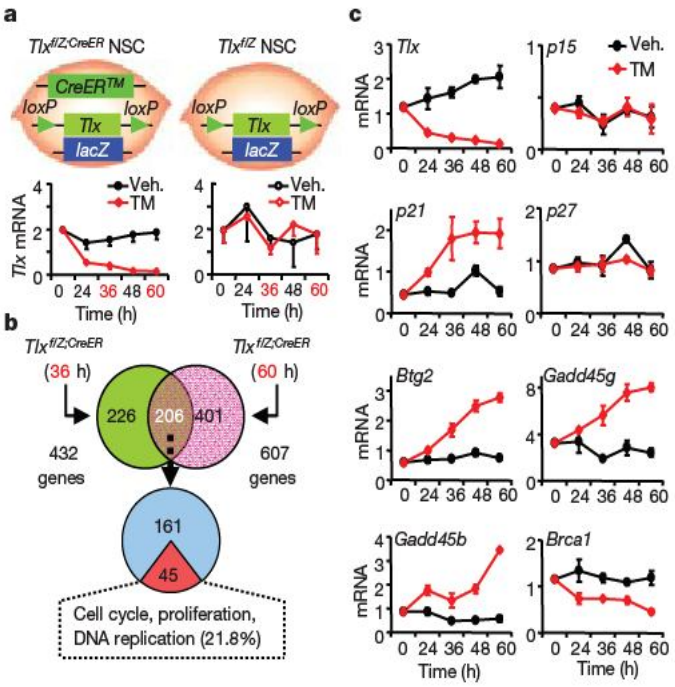

Figure 2 | TLX-regulated genetic programme in adult NSCs. **a**, Tamoxifen-induced, time-dependent deletion of *Tlx* in adult male *Tlx*$^{f/Z;CreER}$ NSCs but not in cells without the CreER transgene. Total RNAs collected at 36 h and 60 h after treatment were used for global gene expression profiling. TM, tamoxifen; veh., vehicle. *x*-axis, time (h) after treatment; *y*-axis, relative expression of mRNA after normalization to that of *hprt*. **b**, Schematic diagram showing TLX-dependent gene expression. Of the 206 genes that changed expression at both time points, 21.8% are implicated in regulation of cell proliferation based on gene ontology analysis. **c**, Quantitative RT–PCR analysis of selected genes for which expression is dependent on TLX. *p15* and *p27* expression were used as controls. Mean ± s.e.m.; $n = 3$.

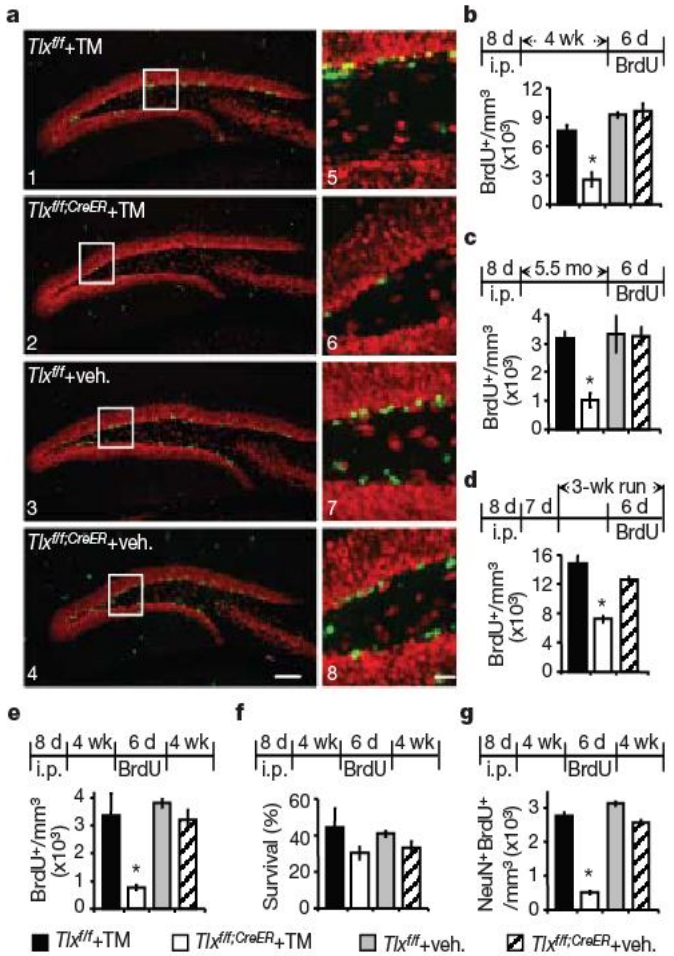

Figure 3 | Deficient NSC proliferation and neurogenesis in the adult hippocampus after inducible deletion of *Tlx*. **a**, Representative coronal sections of the dentate gyrus stained with neuronal marker NeuN (red) and proliferating marker BrdU (green) 4 weeks after 8-day tamoxifen (TM) or vehicle (veh.) treatment of 8-week-old male mice. Panels 5–8 are images taken from boxed regions in panels 1–4, respectively. Scale bar: 1–4, 100 μm; 5–8, 20 μm. **b–d**, BrdU-labelled cells normalized to the volume of the dentate gyrus after 4 weeks (**b**, asterisk, $P < 0.0001$), 5.5 months (**c**, asterisk, $P < 0.003$) or running for 3 weeks (**d**, asterisk, $P < 0.001$) after treatment. **e**, Surviving BrdU-positive cells at 4 weeks. Asterisk, $P < 0.002$. **f**, Survival rate, represented as the ratio of BrdU-positive cells at 4 weeks to that at 1 day after BrdU injection. **g**, Four-week-old new neurons. Asterisk, $P < 0.002$. Mean ± s.e.m.; $n = 4$ for each group. d, day; wk, week; mo, month; i.p., intraperitoneal injection of tamoxifen or vehicle.

was comparable to their controls after three training sessions (Fig. 4a, $F_{1,16} = 0.34$, $P = 0.57$; Supplementary Fig. 5a; two-way analysis of variance (ANOVA) with repeated measures); they also maintained normal contextual memory after 24 h and 48 h (Fig. 4b, $F_{1,16} = 0.13$, $P = 0.73$ at 24 h and $F_{1,16} = 0.74$, $P = 0.40$ at 48 h; Supplementary Fig. 5b; two-way ANOVA with repeated measures). Tone-cued memory measured 24 h later was also similar between treatment groups (Fig. 4c, $P = 0.37$; see also Supplementary Fig. 5c; single factor ANOVA).

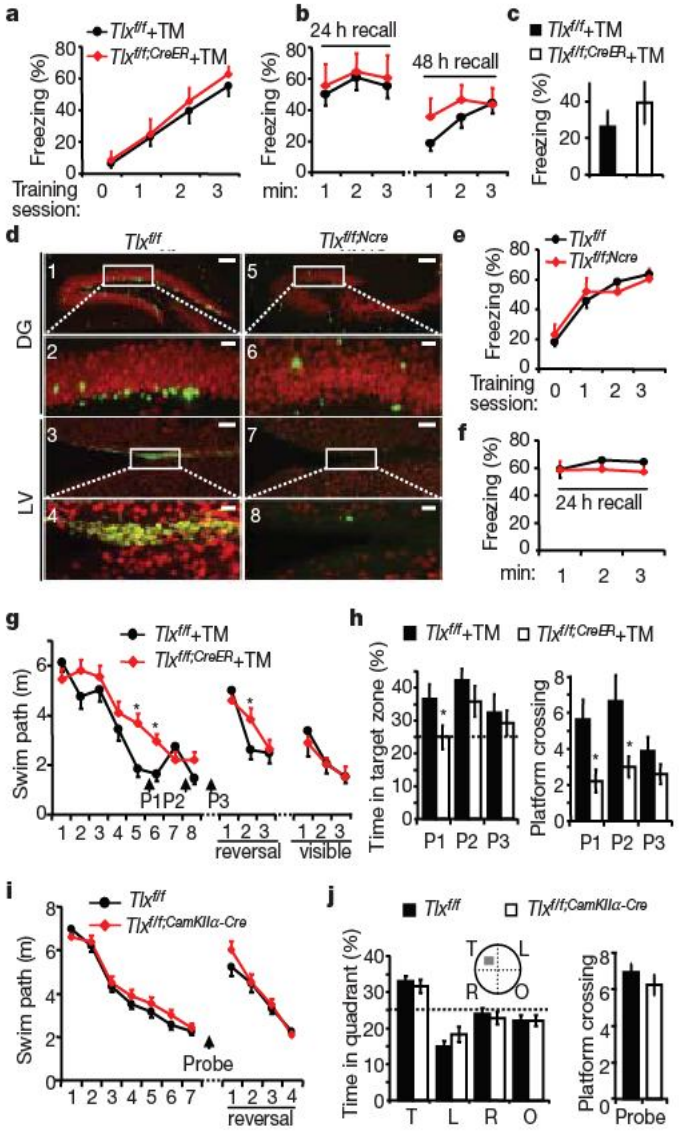

Figure 4 | Normal contextual fear conditioning but impaired spatial learning and memory for mice with defective adult neurogenesis. **a–f**, New neurons are dispensable for contextual fear conditioning. **a–c**, Freezing behaviour monitored during training (**a**), contextual (**b**) or tone-cued memory tests (**c**). $n \geq 8$. **d**, Confocal images showing proliferating cells (BrdU, green). DG, dentate gyrus; LV, lateral ventricle. Panels 2, 4, 6 and 8 are higher magnification views (scale, 20 μm) of the regions boxed in panels 1, 3, 5 and 7 (scale, 100 μm), respectively. **e**, **f**, Freezing behaviour monitored during training (**e**) and contextual memory tests (**f**). $n \geq 14$. **g**, **h**, New neurons in the hippocampus participate in spatial learning. Mice were trained on the water maze (8-day training period) and tested for memory by two probe trials at 12 h (P1, P2) and one probe trial at 3 weeks (P3) after the previous training, followed by 3 days of reversal training and three blocks of training with a visible platform. Dotted line indicates chance level (25%). $n \geq 8$. Asterisk, $P < 0.05$. **i**, **j**, Neuronal deletion of *Tlx* ($Tlx^{f/f;CamKII\alpha\text{-}Cre}$) has no effect on spatial learning and memory. Mice were trained on the water maze, followed by a probe trial at 2 days after the last training and 4-day reversal trainings. $n \geq 28$. T, target zone; L, left zone; R, right zone; O, opposite zone. All data are presented as mean ± s.e.m.

To examine further this unexpected finding, we generated $Tlx^{f/f;NCre}$ mice by crossing the conditional allele to a transgenic line carrying the Cre gene under the control of a nestin enhancer that directs expression in the developing central nervous system (CNS)[17]. This early deletion of *Tlx* leads to a hypomorphic dentate gyrus and an absence of BrdU-labelled cells in adult mutant brains (Fig. 4d; see also Supplementary Fig. 6a, b). Unlike the germline deletion of *Tlx* that results in blindness[9], which may contribute to some of the observed behavioural defects[18], visual function was spared in 2- to 4-month-old $Tlx^{f/f;NCre}$ mice (Supplementary Fig. 6c–g). Although profoundly impaired in spatial learning and memory (Supplementary Figs 7 and 8), $Tlx^{f/f;NCre}$ mice learned and recalled contextual fear conditioning as well as their littermate controls (Fig. 4e, f, $F_{1,34} = 0.00$, $P = 0.97$ for training and $F_{1,34} = 0.30$, $P = 0.59$ for contextual memory; two-way ANOVA with repeated measures). Of note, our protocol can clearly detect the deficit in contextual fear conditioning using a mouse model with normal adult neurogenesis (our own unpublished data). Even when a new cohort of mice was examined using an alternative protocol[15,16], we did not detect a deficit in this behavioural paradigm (Supplementary Fig. 9). Consistent with our data, it has been reported that partial or complete hippocampus-lesioned animals are able to learn and recall as well as controls in contextual fear conditioning[19–21]. Perhaps the observed impairment in contextual fear conditioning in those studies[15,16] is attributable to differences in species or to the side effects of the methods used to knock down neurogenesis[6,22,23].

To test whether adult neurogenesis is involved in spatial learning and memory, we examined a new cohort of mice 4 weeks after an 8-day tamoxifen or vehicle treatment in a more challenging version of the Morris water maze, which uses 40-s learning trials without pre-training. Although tamoxifen-treated $Tlx^{f/f;CreER}$ mice performed similarly in the initial phase of learning (day 1 to day 4) and were able to eventually catch up to the controls (overall performance $F_{1,16} = 3.41$, $P = 0.08$; two-way ANOVA with repeated measures), planned comparisons of swim path revealed significant delays in learning on the fifth and sixth day (Fig. 4g). Indeed, probe trials carried out 12 h after the fifth day session demonstrated a major deficiency in short-term memory for these mutant mice, measured by time in the target zone or platform crossings (Fig. 4h, P1). They also made fewer crosses through the platform location during a second probe trial, indicating a less robust search strategy (Fig. 4h, P2). However, long-term memory examined 3 weeks after the last training session was not affected (Fig. 4h, P3). Notably, tamoxifen-treated $Tlx^{f/f;CreER}$ mice continued to show delayed learning on the second day of a reversal training, as revealed by planned comparisons, suggesting that they could not efficiently associate previous experience with the new task and/or were deficient in cue discrimination (Fig. 4g, reversal 1–3). Such learning and memory impairments are probably not due to a lack of motivation, visual disability or locomotor dysfunction, as these mice performed as well as their littermate controls on a visible version of the water maze, swim velocity, visual cliff, light/dark transition and circadian activity tests (Fig. 4g, visible 1–3; see also Supplementary Figs 10 and 11).

Because tamoxifen-induced recombination also occurred in mature neurons in the dentate gyrus and parts of the cortex (Supplementary Fig. 2a), where *Tlx* shows sporadic expression (Supplementary Fig. 12b, c), the above observed delay in spatial learning may come from the neuronal function of TLX. To examine this possibility, we first analysed the neuronal cell types in which *Tlx* has expression by staining brain sections and found that *Tlx* is specifically expressed in excitatory neurons but not in inhibitory interneurons (Supplementary Fig. 13). This observation was confirmed by immunostaining of cultured hippocampal neurons from postnatal day 0 pups (Supplementary Fig. 14). To delete *Tlx* in these neurons, we crossed $Tlx^{f/f}$ mice to a *CaMKIIα-Cre* transgenic line, which gives rise to postnatal, excitatory neuron-specific recombination[24]. Careful

examination throughout the mouse brains indicated that sparsely expressed *Tlx* cells are found in regions overwhelmingly positive for *CaMKIIα-Cre*-mediated recombination (Supplementary Fig. 12). As expected[24], this strategy gives rise to efficient recombination of *Tlx* in regions expressing *CaMKIIα-Cre*, such as dentate gyrus, CA1 and cortex (see Supplementary Fig. 15). Furthermore, proliferation of NSCs is not affected (data not shown), suggesting that the neuronal function of TLX is not required for NSC maintenance. Interestingly, the performance of $Tlx^{f/f;CamKIIα\text{-}Cre}$ mice was the same as their littermate controls in the water maze under the same challenging training protocol (Fig. 4i,j). These data indicate that most, if not all, neuronal 'non-neurogenic' TLX is not required for spatial learning and memory.

Together, these results provide direct genetic support that NSCs can contribute to the spatial learning and memory circuits[4]. Notably, our results do not support several other studies showing that adult neurogenesis does not have a role in spatial learning[15,25,26]. The cause of such a discrepancy may originate from differences in behavioural testing protocols, the animal species, or genetic backgrounds[27]. Regarding studies specifically using mice[15,28], it is intriguing that, besides the longer duration per trial and/or increased trial numbers per day, both of these previous studies trained the mice on the visible platform before testing with the hidden platform. To test whether such a training paradigm makes a difference on the water maze, we trained a new cohort of mice after inducible deletion of *Tlx* using the protocol described[15,29]. The results clearly demonstrated that under such a training protocol the contribution of adult neurogenesis to spatial learning and memory in the water maze is no longer detectable (Supplementary Fig. 16).

Our development of a new, inducible knockout mouse model provides a new and powerful tool to understand better the role of adult neurogenesis in normal behaviour and disease, and should deepen our insight into which of the many facets of brain function are impacted by this progressive and dynamic cell population.

METHODS SUMMARY

The strategy for generating a conditional allele of *Tlx* using the Cre/*loxP* system is schematically shown in Supplementary Fig. 1. Three correctly targeted embryonic stem cell clones were used to generate mutant mice, which were then backcrossed to C57BL/6J for at least two generations. The resulting Cre transgenic mice were used for crossing to the conditional allele of *Tlx* ($Tlx^{f/f}$): *pNestin-Cre* (ref. 17), *pCAGG-CreER*^{TM} (ref. 10) and *pCamKIIα-Cre* (ref. 24), and the resulting strains, were respectively designated $Tlx^{f/f;NCre}$, $Tlx^{f/f;CreER}$ and $Tlx^{f/f;CamKIIα\text{-}Cre}$. Age- and gender-matched littermates were used for behavioural studies. Adult NSCs were isolated from whole-mouse brains as described[7]. Single-factor ANOVA was used for morphological data. ANOVA with repeated measures over time was applied to the behavioural data. Planned comparison tests were used for post-hoc analysis. $P \leq 0.05$ was considered as significant. All values were expressed as mean ± s.e.m.

Full Methods and any associated references are available in the online version of the paper at www.nature.com/nature.

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LETTERS

HIF-independent regulation of VEGF and angiogenesis by the transcriptional coactivator PGC-1α

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Ischaemia of the heart, brain and limbs is a leading cause of morbidity and mortality worldwide. Hypoxia stimulates the secretion of vascular endothelial growth factor (VEGF) and other angiogenic factors, leading to neovascularization and protection against ischaemic injury[1]. Here we show that the transcriptional coactivator PGC-1α (peroxisome-proliferator-activated receptor-γ coactivator-1α), a potent metabolic sensor and regulator[2], is induced by a lack of nutrients and oxygen, and PGC-1α powerfully regulates VEGF expression and angiogenesis in cultured muscle cells and skeletal muscle *in vivo*. *PGC-1α*$^{-/-}$ mice show a striking failure to reconstitute blood flow in a normal manner to the limb after an ischaemic insult, whereas transgenic expression of PGC-1α in skeletal muscle is protective. Surprisingly, the induction of VEGF by PGC-1α does not involve the canonical hypoxia response pathway and hypoxia inducible factor (HIF). Instead, PGC-1α coactivates the orphan nuclear receptor ERR-α (oestrogen-related receptor-α) on conserved binding sites found in the promoter and in a cluster within the first intron of the *VEGF* gene. Thus, PGC-1α and ERR-α, major regulators of mitochondrial function in response to exercise and other stimuli, also control a novel angiogenic pathway that delivers needed oxygen and substrates. PGC-1α may provide a novel therapeutic target for treating ischaemic diseases.

Ischaemia is a profound metabolic challenge with potentially catastrophic consequences. PGC-1α is a potent modulator of oxidative metabolism in numerous settings[2]. In particular, PGC-1α powerfully regulates oxidative phosphorylation, mitochondrial biogenesis, and respiration[3,4]. To investigate a possible role for PGC-1α in ischaemia, cultured C2C12 myotubes were deprived of nutrients and maintained in 0.2% oxygen. This led within 3 h to an induction of more than tenfold in *PGC-1α* mRNA, as measured by quantitative PCR (Fig. 1a), and an increase in PGC-1α protein, as detected by western blotting (Supplementary Fig. 1). The expression of *PGC-1α* remained elevated when treatment was continued for 48 h (Supplementary Fig. 2) and returned to baseline within 3 h after restoration of complete medium and 21% oxygen (Fig. 1a). This induction of *PGC-1α* was not unique to C2C12 myotubes and was observed in nearly all cell lines tested, including fibroblasts, striatal neurons, hepatocytes and primary skeletal muscle cells (Supplementary Fig. 3). Either removal of nutrients or placement in 0.2% oxygen alone led to a threefold induction of *PGC-1α* expression, whereas the combined treatment led to a synergistic 12-fold induction (Fig. 1b). Hypoxia is well known to induce the expression of a broad genetic programme, at least in part through stabilization of the transcription factors HIF-1α and HIF-2α (ref. 5). This programme includes angiogenic genes such as *VEGF* and glycolytic genes such as *GLUT1*. To examine whether

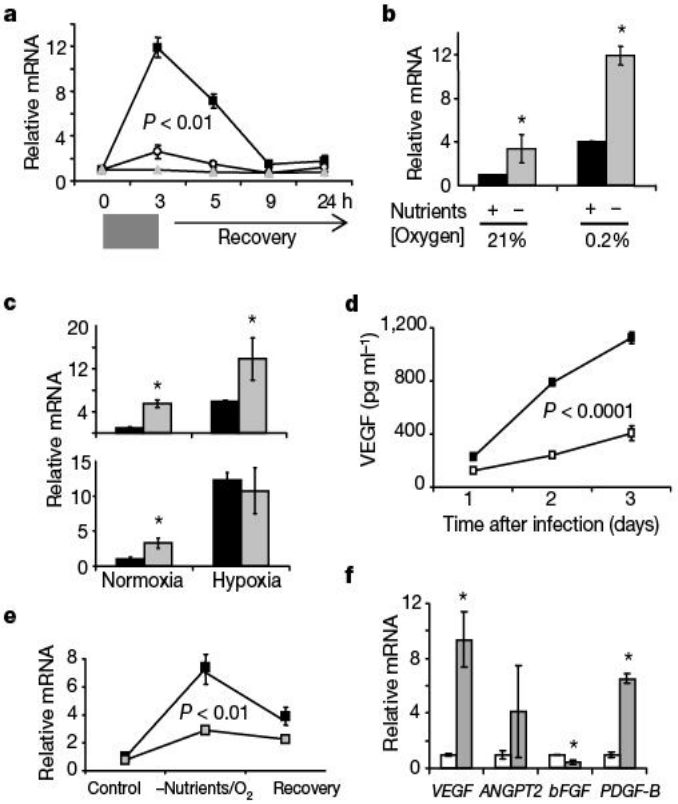

Figure 1 | PGC-1α regulates VEGF in response to deprivation of nutrients and oxygen. **a**, C2C12 myotubes were deprived of oxygen (0.2%) and nutrients for 3 h and then restored to complete medium. Gene expression was measured by quantitative PCR. Squares, *PGC-1α*; circles, *PGC-1β*; triangles, *PRC* (PGC-1-related gene). **b**, Myotubes were deprived of oxygen or nutrients, or both, for 3 h, and expression of *PGC-1α* mRNA was measured. **c**, Myotubes were infected with adenovirus expressing GFP (black bars) or PGC-1α (grey bars) for 24 h and then maintained in either 21% or 0.2% oxygen for 16 h. Top, *VEGF* mRNA; bottom, *GLUT1 mRNA*. **d**, Myotubes were infected as in **c**, and VEGF in the culture medium was measured. Open squares, GFP; filled squares, PGC-1α. **e**, Primary muscle cells derived from *PGC-1α* wild-type (black squares) and knockout (grey squares) animals were treated as in **a**; expression of *VEGF* mRNA was measured. **f**, Primary myotubes were infected with adenovirus expressing GFP (white bars) or PGC-1α (grey bars) and expression of the indicated mRNAs was measured. ANGPT2, angiopoietin-2; bFGF, basic fibroblast growth factor. Error bars indicate s.e.m.; $n > 3$ per group in all panels. Asterisk, $P < 0.05$ compared with control.

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PGC-1α contributes to the regulation of this programme, C2C12 myotubes were infected with adenovirus expressing PGC-1α or green fluorescent protein (GFP) as control. PGC-1α expression led to a fourfold induction of *VEGF* mRNA, which was further increased to 14-fold in the presence of 0.2% oxygen (Fig. 1c and Supplementary Fig. 4). The induced *VEGF* transcript is functional, as demonstrated by an increased secretion of VEGF protein into the cell culture medium (Fig. 1d). All alternatively spliced forms of *VEGF* were induced by PGC-1α (Supplementary Fig. 5). To determine whether PGC-1α is required for the induction of *VEGF*, primary skeletal muscle cells were acquired from *PGC-1α*$^{-/-}$ and wild-type animals. Treating these cells for 3 h with 0.2% oxygen and nutrient deprivation led to an 8-fold induction of *VEGF* in wild-type cells, and this was reduced to threefold in *PGC-1α*$^{-/-}$ cells (Fig. 1e). The induction of *GLUT1* was similarly blunted in *PGC-1α*$^{-/-}$ cells (Supplementary Fig. 6). Hence, under these conditions, PGC-1α contributes greatly to the *VEGF* gene response to nutrient and oxygen deprivation. Angiogenesis in response to hypoxia or other insults is a complex programme, requiring coordination by multiple signals[6]. VEGF has a dominant function in recruiting the endothelium, whereas platelet-derived growth factor (PDGF)-BB recruits mural cells to support and encase the endothelium. Angiopoietin 2, in the presence of VEGF, facilitates the sprouting of new vessels from existing vessels. All three of the genes encoding these proteins are inducible by PGC-1α, as shown by the adenoviral delivery of PGC-1α to primary skeletal muscle cells (Fig. 1f). Together, these data show that PGC-1α is markedly induced by nutrient and oxygen deprivation and, in turn, regulates a wide programme of genes involved in the coordination of neovascularization.

We have previously described transgenic animals that express PGC-1α in some skeletal muscle beds at levels that are about tenfold those in wild-type controls[7]. To test whether PGC-1α can elicit neovascularization *in vivo*, we examined the skeletal muscle of these animals, compared with wild-type littermate controls. Expression of VEGF was induced in all muscle beds examined in the transgenic animals (Fig. 2a, top panel), except in the fibre type I-rich soleus muscle; the transgene is less well expressed in soleus and there is little induction of PGC-1α above wild-type levels (data not shown). Expression of other angiogenic factors, including *PDGF-B* and *angiopoietin 2*, was also induced in transgenic skeletal muscle (Fig. 2a, bottom panel). The density of capillaries was induced markedly in multiple muscle beds of transgenic animals in comparison with wild-type littermate controls, as determined by staining for the endothelial marker CD31 (Fig. 2b and Supplementary Fig. 7). The number of capillaries per high-power field increased from 100 to 300, and the number of capillaries per fibre similarly increased from five to ten. Hence, PGC-1α powerfully induces angiogenesis *in vivo*.

We next tested whether PGC-1α was required for the normal vascular response of skeletal muscle to ischaemia. *PGC-1α*$^{-/-}$ animals and wild-type controls were subjected to ligation and ablation of the femoral artery. In wild-type animals this procedure causes nearly complete cessation of blood flow to the hind limb. This is then followed by progressive neovascularization and return of blood flow to the limb over the ensuing weeks (ref. 8 and Fig. 2c–e). By contrast, the return of blood flow is severely blunted in *PGC-1α*$^{-/-}$ animals (Fig. 2c, d); only about 30% of blood flow is recaptured in *PGC-1α*$^{-/-}$ animals 14 days after surgery, in contrast with more than 50% in wild-type littermate controls. Moreover, in transgenic animals expressing PGC-1α in skeletal muscle, the return of blood flow after ligation of the femoral artery is markedly accelerated (Fig. 2e and Supplementary Fig. 8): 75% of blood flow is recaptured in these animals within six days after surgery, in

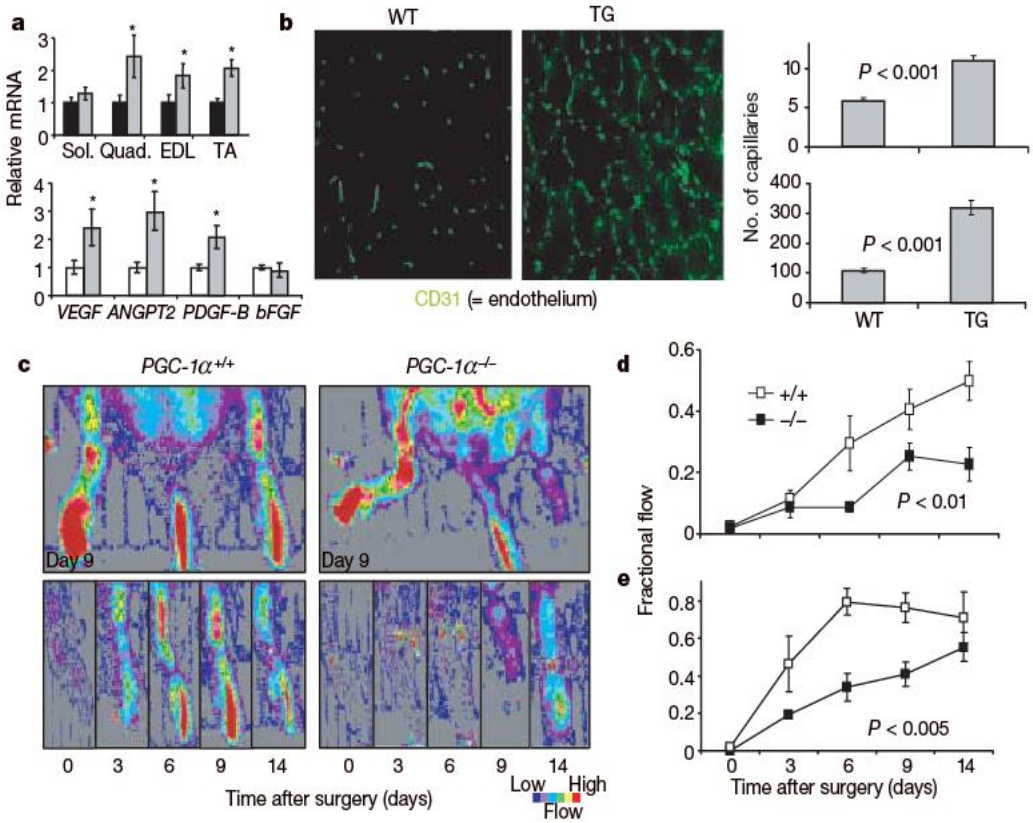

Figure 2 | PGC-1α regulates angiogenesis and blood flow recovery after ischaemia *in vivo*. **a**, Top: expression of *VEGF* mRNA in wild-type (black bars) and muscle creatine kinase (MCK)–PGC-1α transgenic (grey bars) animals. Bottom: expression of the indicated mRNAs in quadriceps from wild-type (white bars) and MCK–PGC-1α transgenic (grey bars) animals. Sol., soleus; quad., quadriceps; EDL, extensor digitorum longus; TA, tibialis anterior. $n = 5$ per group. **b**, Left: transverse sections of TA muscle from wild-type (WT) and transgenic (TG) animals stained for CD31. Right: quantification of capillaries per fibre (top) and per high-power field (bottom). $n = 6$ high-power fields from three mice per group. **c**, **d**, *PGC-1α*$^{+/+}$ and *PGC-1α*$^{-/-}$ mice subjected to femoral artery ligation were subsequently monitored by infrared imaging. Sample images are shown in **c**, and quantification of blood-flow recovery is shown in **d**. $n = 8$ per group. **e**, Blood flow recovery after treating WT (filled squares) and MCK–PGC-1α TG (open squares) mice as in **c**. $n = 6$ per group. Error bars indicate s.e.m. Asterisk, $P < 0.05$ compared with control.

contrast with less than 40% in wild-type controls. Hence, under these conditions, PGC-1α contributes significantly to the physiological response to hindlimb ischaemia. PGC-1α does not seem to affect functional VEGF signalling in endothelial cells (Supplementary Figs 9 and 10), which is consistent with a primary role for PGC-1α in skeletal muscle cells.

The regulation of *VEGF* in response to hypoxia is thought to be mediated primarily through the transcription factors HIF-1α and HIF-2α (ref. 9). However, the regulation of *VEGF* by PGC-1α does not seem to involve this pathway. The hypoxic response element (HRE) that is targeted by HIF is well characterized and, when placed upstream of a luciferase reporter gene, can be activated by the addition of HIF-1α or HIF-2α, or by hypoxia itself. However, the addition of PGC-1α has no effect on this activity, even if tested in the presence of exogenous HIF-1α or HIF-2α, or at reduced oxygen tension (Supplementary Fig. 11). The same is true if the HRE is tested in the context of a larger fragment of the *VEGF* promoter (Supplementary Fig. 12). Delivery of PGC-1α to cells by means of adenovirus has no effect on *HIF-1α* gene expression or protein stability, even though *VEGF* is robustly induced (Fig. 3a). HIF-1α is also not altered in *PGC-1α*$^{-/-}$ cells (Supplementary Fig. 13). HIF-1α and HIF-2α form obligate heterodimers with the aryl hydrocarbon receptor nuclear translocator (ARNT) subunit of HIF, and in cells lacking ARNT there is no HIF activity[10]. However, when PGC-1α is delivered by means of adenovirus to cells lacking ARNT, a fourfold induction of *VEGF* is still seen, equivalent to that seen in wild-type control cells (Fig. 3b). Hence, the induction of *VEGF* by PGC-1α does not require the HIF pathway.

Similarly, the induction of *PGC-1α* by the deprivation of nutrients and oxygen is apparently independent of HIF. The promoter region of *PGC-1α*, placed upstream of a luciferase reporter gene, is not affected by the addition of a constitutively active form of HIF-1α, whereas the promoter region of *VEGF* is strongly activated (Supplementary Fig. 14). A number of small molecules, including $CoCl_2$, deferroxamine and dimethyloxallyl glycine, can activate the HIF pathway and induce *VEGF*, *GLUT1* and other target genes; these molecules, however, have no effect on *PGC-1α* expression (Fig. 3c and Supplementary Fig. 15). Finally, the induction of *PGC-1α* by the deprivation of nutrients and oxygen is intact in cells lacking ARNT (Fig. 3d, top panel, and Supplementary Fig. 16), whereas the induction of canonical HIF targets is blunted (Fig. 3d, bottom panel, and Supplementary Fig. 17). Together, these data clearly show that PGC-1α has a critical function in an angiogenic pathway that is independent of the canonical HIF pathway.

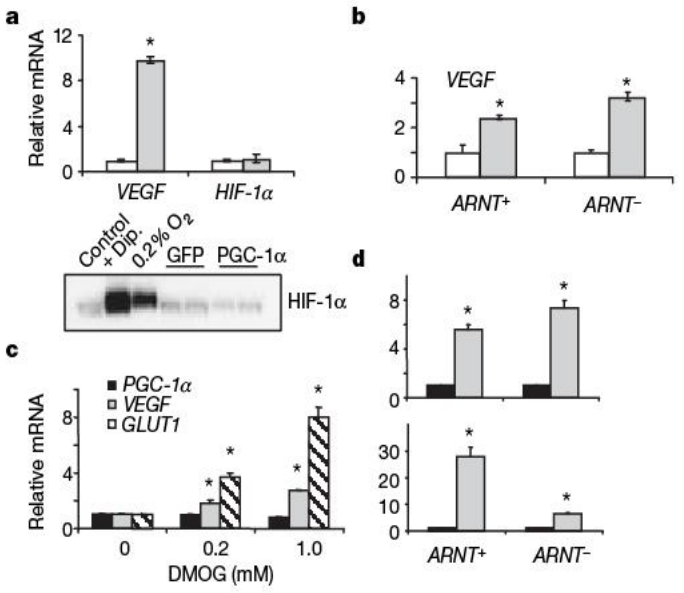

Figure 3 | PGC-1α is induced by nutrient/oxygen deprivation and regulates VEGF independently of the HIF pathway. a, Primary skeletal muscle cells were infected with adenovirus expressing GFP (white bars) or PGC-1α (grey bars). After 48 h, *HIF-1α* mRNA (top), and protein (bottom) was measured. HIF-1α protein levels after treatment for 6 h with dipyridamole (dip.) or hypoxia (0.2% O_2) are shown as controls. ct, control. **b**, *ARNT*$^-$ and *ARNT*$^+$ cells were infected with adenovirus expressing GFP (white bars) or PGC-1α (grey bars), and *VEGF* mRNA was measured. **c**, C2C12 myotubes were treated with dimethyloxallyl glycine (DMOG) for 24 h, and expression of the indicated mRNAs was measured. **d**, *ARNT*$^-$ and *ARNT*$^+$ cells were deprived of oxygen and nutrients for 3 h (grey bars), and expression of *PGC-1α* mRNA (top) and *VEGF* mRNA (bottom) was measured. Black bars, controls. Error bars indicate s.e.m.; $n > 3$ per group in all panels. Asterisk, $P < 0.05$ compared with control.

PGC-1α is known to co-activate several transcription factors, including many members of the MEF2 (myocyte enhancer factor-2), FOXO (forkhead transcription factor O) and nuclear receptor families[2]. Plasmids expressing several of these transcription factors, with or without PGC-1α, were co-transfected into 10T1/2 cells along with a reporter plasmid containing 2 kilobases of the *VEGF* promoter driving the luciferase gene. The addition of PGC-1α to nuclear respiratory factor (NRF)-1 or NRF-2 (Fig. 4a), MEF2s or FOXO1 (data not shown) had no effect on luciferase activity. The addition of PGC-1α to the orphan nuclear receptor ERR-α, in contrast, led to an eightfold induction of luciferase activity (Fig. 4a). ERR-α is known to interact physically and functionally with PGC-1α and is involved in the activation of programmes of fatty acid oxidation and oxidative phosphorylation[11–16]. The induction of luciferase activity by PGC-1α plus ERR-α did not require the integrity of the HIF-responsive element in the *VEGF* promoter (Supplementary Fig. 18). Instead, it required the integrity of two putative ERR-α-binding sequences, both of which are perfectly conserved across vertebrate species (Supplementary Fig. 19). To examine the role of ERR-α in regulating endogenous VEGF, primary skeletal muscle cells were infected with adenovirus expressing ERR-α. This led to a threefold induction of *VEGF* mRNA, compared with infection with a control virus expressing GFP alone (Fig. 4b). As a critical test for whether ERR-α is required for the PGC-1α-mediated induction of *VEGF*, primary mouse embryonic fibroblasts were prepared from *ERR-α*$^{-/-}$ and wild-type animals, and the expression of VEGF was evaluated after infection with adenovirus expressing PGC-α. In wild-type mouse embryonic fibroblasts, PGC-1α induced *VEGF* expression sevenfold, compared with virus expressing GFP alone (Fig. 4c). In sharp contrast, the induction of *VEGF* by PGC-1α was completely abrogated in *ERR-α*$^{-/-}$ mouse embryonic fibroblasts. The induction of *PDGF-B* by PGC-1α was also abrogated in these cells (Supplementary Fig. 20). Consistent with these findings is our observation that the expression of *VEGF* in *ERR-α*$^{-/-}$ primary skeletal muscle cells was reduced by about 50% (Supplementary Fig. 21). Thus, PGC-1α seems to stimulate VEGF expression at least in part by the coactivation of ERR-α.

ERR-α recognizes the consensus DNA sequence AAGGTCA[17]. A search through the 25 kilobases surrounding the murine *VEGF* gene revealed the existence of 11 such sites, whereas only three or four would have been predicted by chance alone (Fig. 4d). Of these 11 sites, 6 are perfectly conserved between human, mouse and rat (red arrows in Fig. 4d); strikingly, 5 of them are clustered within regions of high homology in the first intron of the *VEGF* gene. A 1,200-base-pair region encompassing these five sites was amplified by polymerase chain reaction and cloned upstream of the *SV40* promoter and a luciferase reporter gene. Co-transfection of *PGC-1α* and *ERR-α* with this reporter plasmid led to a synergistic sevenfold induction of luciferase activity (Fig. 4e, left half). By contrast, PGC-1α and ERR-α had no effect on a control plasmid containing the *SV40* promoter alone (Fig. 4e, right half). The induction of luciferase activity was dependent on the integrity of the conserved ERR-α sites (Supplementary Fig. 22). Chromatin immunoprecipitation assays revealed that PGC-1α can occupy these ERR-α sites as well as the ERR-α sites found on the promoter (Fig. 4f). Thus, the first intron of the *VEGF* gene contains a putative enhancer region in which several conserved

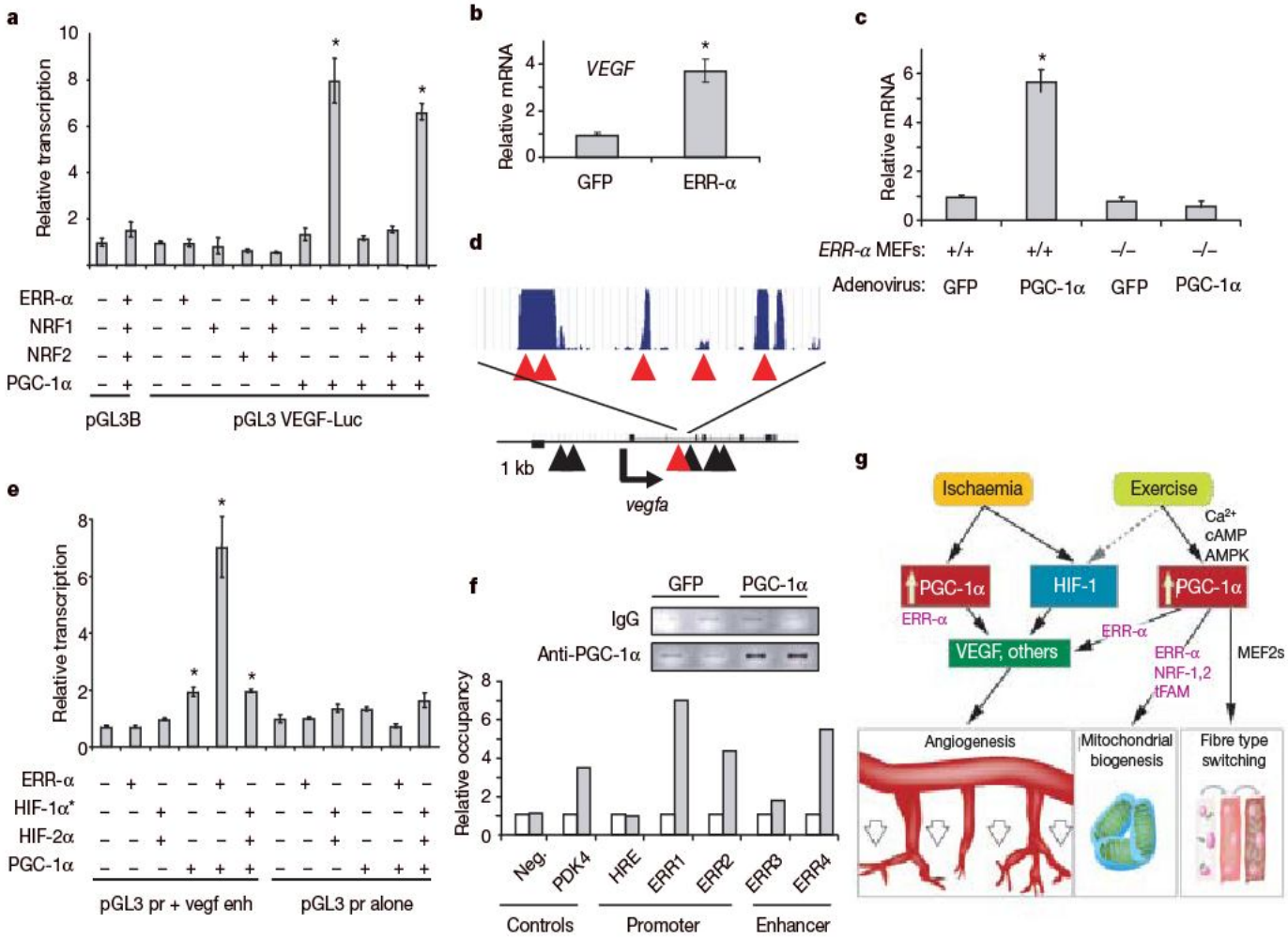

Figure 4 | PGC-1α regulates expression of VEGF through coactivation of ERR-α. **a**, luciferase (Luc) activity in cells transfected with VEGF or control promoter constructs, plus vectors expressing the indicated proteins. **b**, *VEGF* mRNA in primary skeletal myotubes infected with adenovirus expressing GFP or PGC-1α. **c**, *ERR-α*$^{+/+}$ and *ERR-α*$^{-/-}$ mouse embryonic fibroblasts evaluated as in **b**. **d**, Diagram of the murine *vegfa* gene region. Arrowheads indicate consensus ERR-α binding sites; red arrowheads indicate sites perfectly conserved between mammals. kb, kilobase. The histogram shows interspecies homology. See Supplementary Fig. 15 for details. **e**, Luciferase activity in cells transfected with the indicated plasmids, plus a reporter construct containing the region depicted in **d**. pr, SV40 promoter; vegf enh, *vegf* enhancer. **f**, Chromatin immunoprecipitations in C2C12 myotubes infected with the indicated adenovirus. White bars, IgG; grey bars, PGC-1α. ERR1–ERR4, putative ERR-α-binding sites in the *VEGF* promoter and enhancer (see Supplementary Figs 13 and 15); PDK4, known site in the *PDK4* promoter; HRE, hypoxic responsive element; neg., unrelated site. **g**, Speculative model for the role of PGC-1α in the regulation of angiogenesis during exercise and in response to ischaemia. AMPK, AMP-activated protein kinase; tFAM, mitochondrial transcription factor A. Error bars indicate s.e.m. Asterisk, $P < 0.05$ compared with control.

ERR-α-binding sites are recognized by ERR-α and coactivated by PGC-1α to elicit the robust induction of VEGF transcription.

The data here show that PGC-1α is a mediator of signalling in response to deprivation of nutrients and oxygen, and that it powerfully regulates *VEGF* and other angiogenic factors to elicit neovascularization *in vivo*. The regulation of VEGF in response to hypoxia is thought to be mediated primarily through the well-known HIF factors[9]. Surprisingly, the novel PGC-1α/ERR-α pathway described here is apparently independent of the HIF pathway. *PGC-1α*$^{-/-}$ mice are viable, suggesting that PGC-1α is not essential in embryonic vascularization. Angiogenesis in the adult occurs in both physiological and pathological contexts[1]. The robust induction of vascularization by PGC-1α, and its critical function in the response to limb ischaemia, strongly implicate PGC-1α in the angiogenic response to ischaemia, providing protection against further ischaemic insults (Fig. 4g). PGC-1α is also robustly induced by exercise and mediates known responses to exercise such as fibre-type switching and mitochondrial biogenesis[3,7]. On the basis of these observations and the data presented here, we speculate that the PGC-1α/ERR-α pathway also mediates exercise-induced neovascularization (Fig. 4g). This elegantly links the regulation of consumption of oxygen by mitochondria to the delivery of oxygen and nutrients by the vasculature. Angiogenesis is also crucial to tumour progression and metastasis. The interface of metabolism with cancer progression has been the subject of renewed scrutiny in recent years[18]. It will be of great interest to elucidate the role of PGC-1α and ERR-α in this interface, given the important function of these molecules in metabolic control.

Human clinical trials that examine the efficacy of VEGF delivery as therapy in various settings, including chronic limb ischaemia, have yielded disappointing results[6,19,20]. In large part this may be because the use of VEGF alone seems to lead to immature, leaky vessels[19]. The generation of fully functional vessels requires the coordinated action of numerous signals, such as PDGF-BB and the angiopoetins[6]. One therapeutic approach to this problem may be to modulate a transcriptional regulator that coordinates these signals appropriately[6,21]. The PGC-1α/ERR-α pathway provides such an opportunity.

METHODS SUMMARY

Nutrient and oxygen deprivation was induced by placing cells in Hanks balanced salt solution and 0.2% oxygen. Hindlimb ischaemia was induced by ligation of the femoral artery, as described[8]. Infections with adenovirus and transfections were performed as described[22]. One-way analysis of variance with repeated values was used to analyse data from femoral ligations. Two-tailed independent Student's *t*-tests were used to determine all other *P* values.

Full Methods and any associated references are available in the online version of the paper at www.nature.com/nature.

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LETTERS

A PtdIns4,5P_2-regulated nuclear poly(A) polymerase controls expression of select mRNAs

David L. Mellman[1]*, Michael L. Gonzales[1]*, Chunhua Song[2]*, Christy A. Barlow[2]*, Ping Wang[3], Christina Kendziorski[3] & Richard A. Anderson[1,2]

Phosphoinositides are a family of lipid signalling molecules that regulate many cellular functions in eukaryotes. Phosphatidylinositol-4,5-bisphosphate (PtdIns4,5P_2), the central component in the phosphoinositide signalling circuitry, is generated primarily by type I phosphatidylinositol 4-phosphate 5-kinases (PIPKIα, PIPKIβ and PIPKIγ)[1]. In addition to functions in the cytosol, phosphoinositides are present in the nucleus[2,3], where they modulate several functions[4–6]; however, the mechanism by which they directly regulate nuclear functions remains unknown. PIPKIs regulate cellular functions through interactions with protein partners, often PtdIns4,5P_2 effectors, that target PIPKIs to discrete subcellular compartments, resulting in the spatial and temporal generation of PtdIns4,5P_2 required for the regulation of specific signalling pathways[1,7]. Therefore, to determine roles for nuclear PtdIns4,5P_2 we set out to identify proteins that interacted with the nuclear PIPK, PIPKIα. Here we show that PIPKIα co-localizes at nuclear speckles and interacts with a newly identified non-canonical poly(A) polymerase, which we have termed Star-PAP (nuclear speckle targeted PIPKIα regulated-poly(A) polymerase) and that the activity of Star-PAP can be specifically regulated by PtdIns4,5P_2. Star-PAP and PIPKIα function together in a complex to control the expression of select mRNAs, including the transcript encoding the key cytoprotective enzyme haem oxygenase-1 (refs 8, 9) and other oxidative stress response genes by regulating the 3′-end formation of their mRNAs. Taken together, the data demonstrate a model by which phosphoinositide signalling works in tandem with complement pathways to regulate the activity of Star-PAP and the subsequent biosynthesis of its target mRNA. The results reveal a mechanism for the integration of nuclear phosphoinositide signals and a method for regulating gene expression.

To identify nuclear PIPKIα-interacting proteins, the nuclear speckle-targeting region of PIPKIα (amino-acid residues 440–562; Fig. 1a) was used as bait in a yeast two-hybrid screen. Among the interactors identified was a protein we have named Star-PAP. Star-PAP is a member of the DNA polymerase β-like superfamily of nucleotidyltransferases and is most closely related to poly(A) polymerases (PAPs). Star-PAP is unique among known PAPs[10,11] (Fig. 1b) in that it contains a split PAP domain linked by a proline-rich region, an amino-terminal C_2H_2 zinc-finger, an RNA recognition motif, a PAP catalytic and core domain, a PAP-associated domain, an R/S repeat and a nuclear localization signal (Fig. 1b). Star-PAP has putative family members throughout species (Supplementary Fig. 2) and is expressed in numerous cultured cell lines and ubiquitously in humans (Supplementary Fig. 3).

The interaction between Star-PAP and PIPKIα was confirmed by an *in vitro* glutathione *S*-transferase (GST) pull-down assay. Star-PAP bound to GST–PIPKIα and the GST–PIPKIα carboxy terminus, but not to GST alone (Fig. 1c). Immunoprecipitation of endogenous Star-PAP resulted in co-immunoprecipitation of PIPKIα (Fig. 1d) but not other PIPKI isoforms (data not shown), showing that this interaction occurs *in vivo*. Star-PAP localizes at nuclear speckles, as shown by its co-localization with PIPKIα and Sm proteins and by its loss at speckles on RNA-mediated interference (RNAi) knockdown (Fig. 1e, f). The targeting of Star-PAP with PIPKIα and PtdIns4,5P_2, to a compartment where pre-mRNA processing factors and phosphoinositide metabolism are concentrated[3,12] suggested that Star-PAP functions in mRNA biosynthesis and may be regulated by phosphoinositides.

On the basis of the sequence homology between Star-PAP and known poly(A) polymerases (Supplementary Fig. 4) Star-PAP was assayed for PAP activity[13]. Recombinant purified Star-PAP extended an A_{15} RNA primer with [α-^{32}P]ATP (Fig. 2a). As with known PAPs, Star-PAP activity was inhibited by the chain terminator cordycepin triphosphate (0–5.0 mM) (Fig. 2b), and nucleotide incorporation into the RNA substrate by Star-PAP was selective for ATP (Fig. 2c). Moreover, 3′ tails generated with all four rNTPs in the reaction mixture were digested with oligo(dT)/RNase H, indicating that the extension of the RNA primer is primarily through the addition of AMP (Fig. 2d). Mutations of catalytic residues (Supplementary Fig. 4, asterisk) within the nucleotidyl transferase motif[14] enfeebled PAP activity (Fig. 2e). Together these data indicate that Star-PAP possesses PAP activity.

Recently, recombinant Star-PAP purified from HeLa cells was reported to have terminal uridylyl transferase (TUTase) activity specific for U6 snRNA *in vitro*[15]. Under both defined TUTase and PAP assay conditions, Star-PAP has the capacity to transfer UMP residues to RNA (Supplementary Fig. 5). However, the concentration of ATP in cells is much greater than that of UTP[16]. We therefore subjected Star-PAP to an *in vitro* nucleotide competition assay to determine its preferred nucleotide substrate. Star-PAP activity towards UTP was effectively inhibited by the addition of excess ATP, but not vice versa (Supplementary Fig. 5). Although Star-PAP does indeed have genuine TUTase activity, these data show that, with *in vivo* ratios of rNTPs, Star-PAP preferentially uses ATP as a nucleotide substrate, indicating that it functions primarily as a poly(A) polymerase.

Because Star-PAP interacts with PIPKIα, we determined whether Star-PAP is an effector of the PIPKIα product PtdIns4,5P_2. In the presence of 50 μM PtdIns4,5P_2, Star-PAP activity was markedly stimulated, particularly with regard to products more than 200 nucleotides in length (Fig. 2f, h, i). Other phosphoinositides did not affect Star-PAP activity, and no phosphoinositide had an effect on PAPα activity (Fig. 2g, h), demonstrating that PtdIns4,5P_2 stimulation is a unique trait of Star-PAP. In addition, inositol-1,4,5-trisphosphate

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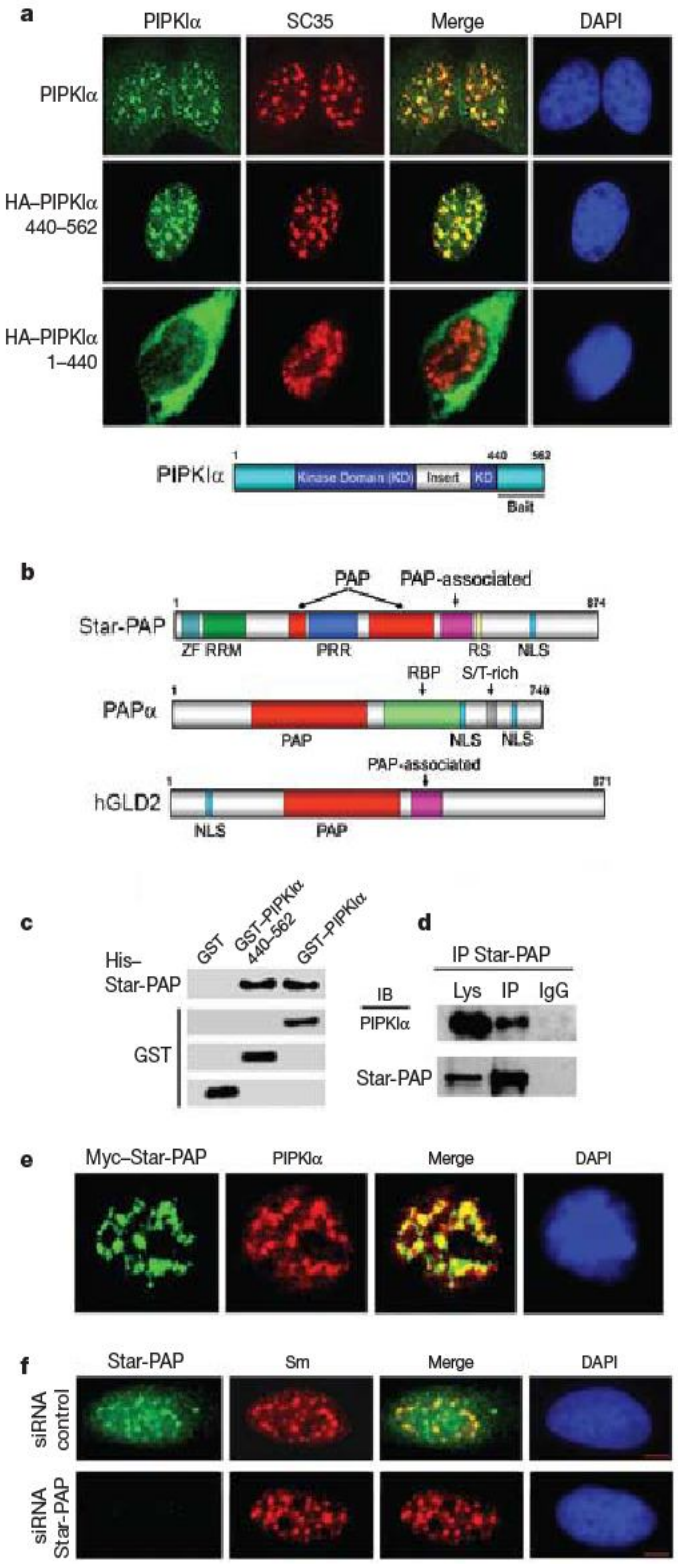

Figure 1 | Identification of a nuclear-localized PIPKIα-interacting protein. **a**, Localization of PIPKIα, haemagglutinin (HA)-tagged PIPKIα C terminus (residues 1–439), HA–PIPKIα N terminus (residues 440–562) (green), SC-35 (red) and 4′,6-diamidino-2-phenylindole (blue) in HeLa cells. The underlined region of the PIPKIα diagram represents bait used in the yeast two-hybrid screen. **b**, Diagram of Star-PAP domain arrangement compared with those of PAPα and human Germ line development 2 (hGld2). NLS, nuclear localization signal; PRR, proline-rich region; RBD, RNA-binding domain; RRM, RNA recognition motif; RS, arginine/serine repeat; ZF, zinc-finger. **c**, *In vitro* GST pull-down with His–Star-PAP and GST–PIPKIα full-length and C terminus. **d**, Immunoprecipitation (IP) of Star-PAP and detection of associated PIPKIα. IB, immunoblot. **e**, Subnuclear localization of Myc-tagged Star-PAP (green) and endogenous PIPKIα (red). **f**, Subnuclear localization of endogenous Star-PAP (green) and Sm (red) after RNAi knockdown with control oligonucleotide (top) or oligonucleotide specific for Star-PAP (bottom). Scale bar, 5 μm.

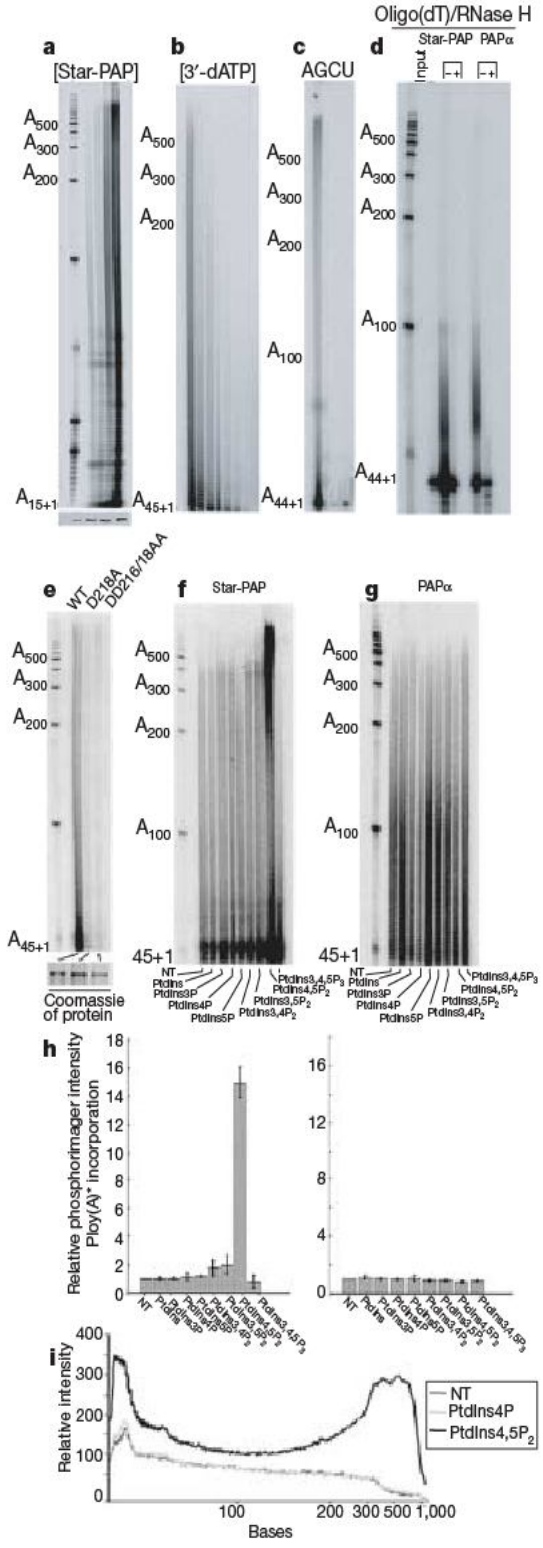

Figure 2 | Star-PAP has poly(A) polymerase activity that is stimulated by PtdIns4,5P$_2$. **a**, The activity of His-Star-PAP (0–1.25 μM) towards A_{15} RNA primer. Anti-T7 western blot (bottom) demonstrates protein levels. **b**, Effects of cordycepin triphosphate on His–Star-PAP activity. **c**, Star-PAP activity towards all four rNTPs. **d**, Oligo(dT)/RNase H treatment of Star-PAP-generated RNA product. **e**, Effects on mutations of conserved catalytic residues in Star-PAP. Coomassie blue stain demonstrates protein levels. **f**, **g**, Effects of 50 μM inositol phospholipid micelles on His–Star-PAP (**f**) and PAPα (1 μM) (**g**) activity. NT, non-treated vehicle-only control. **h**, Incorporation of [α-^{32}P]ATP into poly(A)$^+$ products larger than A_{200} in the presence of phosphoinositide micelles by Star-PAP and PAPα from **f** and **g** ($n = 3$). Error bars represent s.e.m. **i**, Relative distributions of poly(A)$^+$ products from non-treated (NT), PtdIns4P-treated and PtdIns4,5P$_2$-treated Star-PAP from **f**.

did not stimulate Star-PAP activity, demonstrating that this regulation is specific for the lipid species (data not shown).

PAPs associate with protein partners that define their function *in vivo*[11,17–19]. Canonical PAPα associates *in vivo* with factors required for the polyadenylation of mRNA, including cleavage and polyadenylation specificity factor (CPSF) and cleavage stimulation factor (CstF) subunits, symplekin and RNA polymerase II (RNA Pol II)[10,18,20–22]. Similarly, endogenous Star-PAP co-immunoprecipitated with CPSF-73, RNA Pol II and symplekin (Fig. 3a, b). Endogenous PIPKIα specifically associated with Flag-tagged Star-PAP and was able to generate PtdIns4,5P_2 *in vitro* (Fig. 3c), which suggests that in accordance with the model of spatial phosphoinositide generation defining its function, the production of PtdIns4,5P_2 *de novo* occurs in proximity to Star-PAP to regulate its activity *in vivo*. To compare their associated protein complexes, Flag–Star-PAP and Flag–PAPα were expressed and affinity purified in parallel from HEK-293 cells. Both Star-PAP and PAPα associated with mRNA 3′-processing factors (Supplementary Fig. 6). PAPα was not detected in the Star-PAP complex; nor was the reverse true (Fig. 4g and Supplementary Fig. S6); in addition, the exosome components Rrp6 and Rrp46 (ref. 17) were not detected in the Star-PAP complex (data not shown), indicating specificity with pre-mRNA 3′-processing factors. Phylogenetic analysis demonstrates that Star-PAP clusters with PAPs that polyadenylate mRNAs (Supplementary Fig. 7), supporting the hypothesis that Star-PAP functions as a PAP in the 3′-end formation of mRNAs.

Polyadenylation of mRNA is critical for its stability[23,24]. Therefore, a loss of Star-PAP would be predicted to decrease the level of mRNAs that it polyadenylates. Moreover, if PIPKIα has a functional relationship with Star-PAP, knockdown of PIPKIα should cause a decrease in a pool of target mRNAs that require both Star-PAP and PIPKIα for their maturation. To test this, we knocked down Star-PAP or PIPKIα and performed a microarray analysis of total polyadenylated mRNAs from each group. A significant (conditional false discovery rate ≤ 0.01) change in transcript level compared with control cells ($n = 3$) was detected for 4,481 genes with *Star-PAP* RNAi knockdown and 4,542 genes with *PIPKIα* RNAi knockdown. There was an overlap of 2,350 significant gene changes in both conditions, of which 2,262 were in the same direction (Fig. 3d).

A large group of the identified genes encode proteins involved in detoxification and/or oxidative stress response. Some of these, including those encoding haem oxygenase-1 (*HO-1*), NAD(P)H:quinone oxidoreductase 1 (*NQO1*), apolipoprotein E (*APOE*), peroxiredoxin 1 (*PRDX1*), glutathione *S*-transferase κ1 (*GSTK1*) and aldehyde dehydrogenase 2 family (mitochondrial) (*ALDH2*), were chosen for validation by quantitative real-time RT–PCR (qRT–PCR). The expression levels of these candidate mRNAs were consistent with the microarray analysis, demonstrating that Star-PAP is required for the expression of these mRNAs. *PIPKIα* RNAi knockdown also significantly decreased the expression levels of these same mRNAs, indicating that PIPKIα modulates select Star-PAP-dependent gene expression (Fig. 3e). Knockdown of both Star-PAP and PIPKIα showed no additive effect on the loss of *HO-1* or *NQO1* mRNA, providing evidence that Star-PAP and PIPKIα function in a common pathway to control their expression (data not shown).

To determine direct targets of Star-PAP, RNA immunoprecipitation[25] was used. Star-PAP was associated with *HO-1* mRNA but not with the non-target mRNAs encoding glutamate cysteine ligase, catalytic subunit (GCLC) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Fig. 3f). HO-1 is an important cytoprotective enzyme whose expression is achieved primarily through the modulation of its mRNA levels, and the induction of *HO-1* mRNA is a key cellular response to reactive oxygen species and other cellular stresses[8,9]. Because *HO-1* is a direct target of Star-PAP, it was selected for use in exploring the mechanism by which Star-PAP controls the expression of its select target mRNA.

We tested the hypothesis that Star-PAP and PIPKIα are necessary for *HO-1* mRNA expression during an antioxidant response. RNAi knockdown of both Star-PAP and PIPKIα decreased basal levels of *HO-1* mRNA and inhibited the maximal expression of *HO-1* mRNA in response to treatment with 100 μM t-butylhydroquinone (tBHQ)[8]

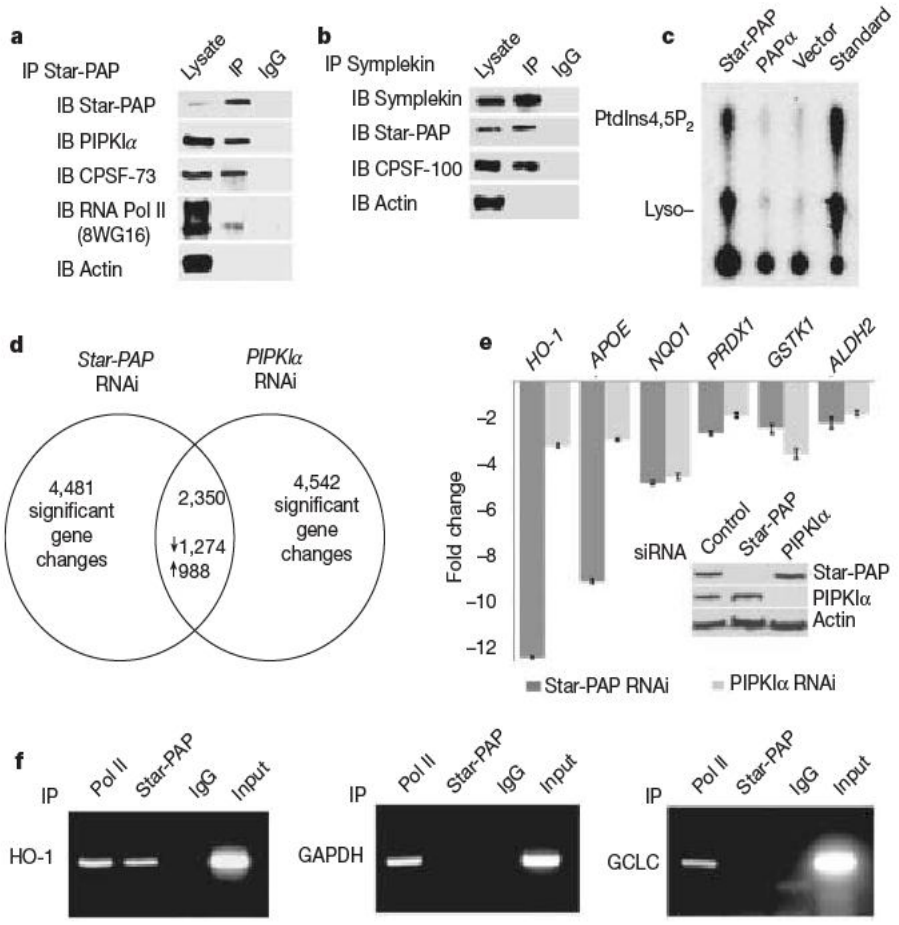

Figure 3 | Star-PAP-interacting proteins and identification of Star-PAP target mRNAs. **a**, Immunoprecipitation (IP) of Star-PAP from HEK-293 cells, followed by western blot analysis (IB) for PIPKIα, CPSF-73 and RNA Pol II (8WG16). **b**, Immunoprecipitation of symplekin from HEK-293 cells followed by western blot analysis for Star-PAP and CPSF-100. **c**, PIP kinase activity of purified PAP complexes using PtdIns4P as substrate. Lyso, PtdIns4,5P_2 degradation product in which the inositol headgroup is lacking one acyl chain. **d**, Venn diagram depicting mRNA expression profiles on *Star-PAP* or *PIPKIα* RNAi knockdown versus control. **e**, qRT–PCR analysis of selected mRNAs from **d** ($n = 3$). Error bars represent s.e.m. **f**, RNA immunoprecipitation of Star-PAP and RNA Pol II (N-20) from HEK-293 cells. Primers are listed in Supplementary Information.

(Fig. 4a). This indicated that Star-PAP and PIPKIα were required for basal and maximal expression, demonstrating that they regulate *HO-1* mRNA levels on induction of oxidative stress.

To demonstrate Star-PAP involvement in the processing and expression of *HO-1* mRNA, Star-PAP and PIPKIα were knocked down by means of RNAi, and the 3′-end formation of *HO-1* mRNA was directly examined in an *in vivo* functional assay. Star-PAP knockdown resulted in a ~20 fold increase in the quantity of uncleaved *HO-1* mRNA relative to total (Fig. 4b, c, e). In contrast, the amount of uncleaved *GCLC* mRNA was not changed by either Star-PAP or PIPKIα knockdown (Fig. 4d, f). This is consistent with reports that PAP is required for efficient 3′ cleavage by the endonuclease CPSF-73 *in vitro*[22,26,27], and indicates that Star-PAP is functioning as a PAP for the maturation of *HO-1* mRNA. PIPKIα knockdown had a smaller affect on *HO-1* mRNA cleavage (Fig. 4b, c, e), consistent with PIPKIα modifying Star-PAP function. The accumulation of unprocessed *HO-1* mRNA on Star-PAP knockdown is consistent with Star-PAP functioning as PAP *in vivo* and demonstrates that Star-PAP is required for efficient 3′-end formation of *HO-1* mRNA.

To explore the mechanism by which Star-PAP acts in the 3′ processing of mRNA, we examined the effect of stimulation of cells by tBHQ on Star-PAP complex assembly. The association of endogenous Star-PAP with PIPKIα, CPSF-73 and RNA Pol II was greatly enhanced by treatment with 100 μM tBHQ for 4 h (Fig. 4g, h). Further, Star-PAP complex purified from stably expressing cells treated with tBHQ showed a more than 15-fold increase in enzymatic activity over Star-PAP from control cells (Fig. 4i). Neither polymerase-inactive Star-PAP nor PAPα showed any increase in activity when isolated from tBHQ-treated cells (Fig. 4i, k). Treatment of cells with tBHQ caused a large increase in Star-PAP complex activity for the initiation of polyadenylation. When Star-PAP was isolated from tBHQ-treated cells, PtdIns4,5P_2 robustly stimulated Star-PAP processivity, increasing the length of the poly(A) tail, as can be seen over a time course (Fig. 4i, j). This demonstrates that tBHQ-induced signalling and PtdIns4,5P_2 modulate Star-PAP activity in two distinct yet complementary manners. These data suggest a model in which an antioxidant response induces the assembly of the Star-PAP complex, leading to a rapid initiation of 3′-end formation and polyadenylation by the Star-PAP complex. PtdIns4,5P_2 produced by PIPKIα in the complex then controls the processivity of Star-PAP, resulting in a lengthened poly(A) tail. In this manner Star-PAP may respond to oxidative stress signals, and potentially other signals, to efficiently regulate the 3′-end formation and expression of its target mRNAs.

Here we have identified and characterized a non-canonical phosphoinositide-sensitive poly(A) polymerase, Star-PAP. PtdIns4,5P_2 regulates Star-PAP processivity and thus controls the 3′-end formation

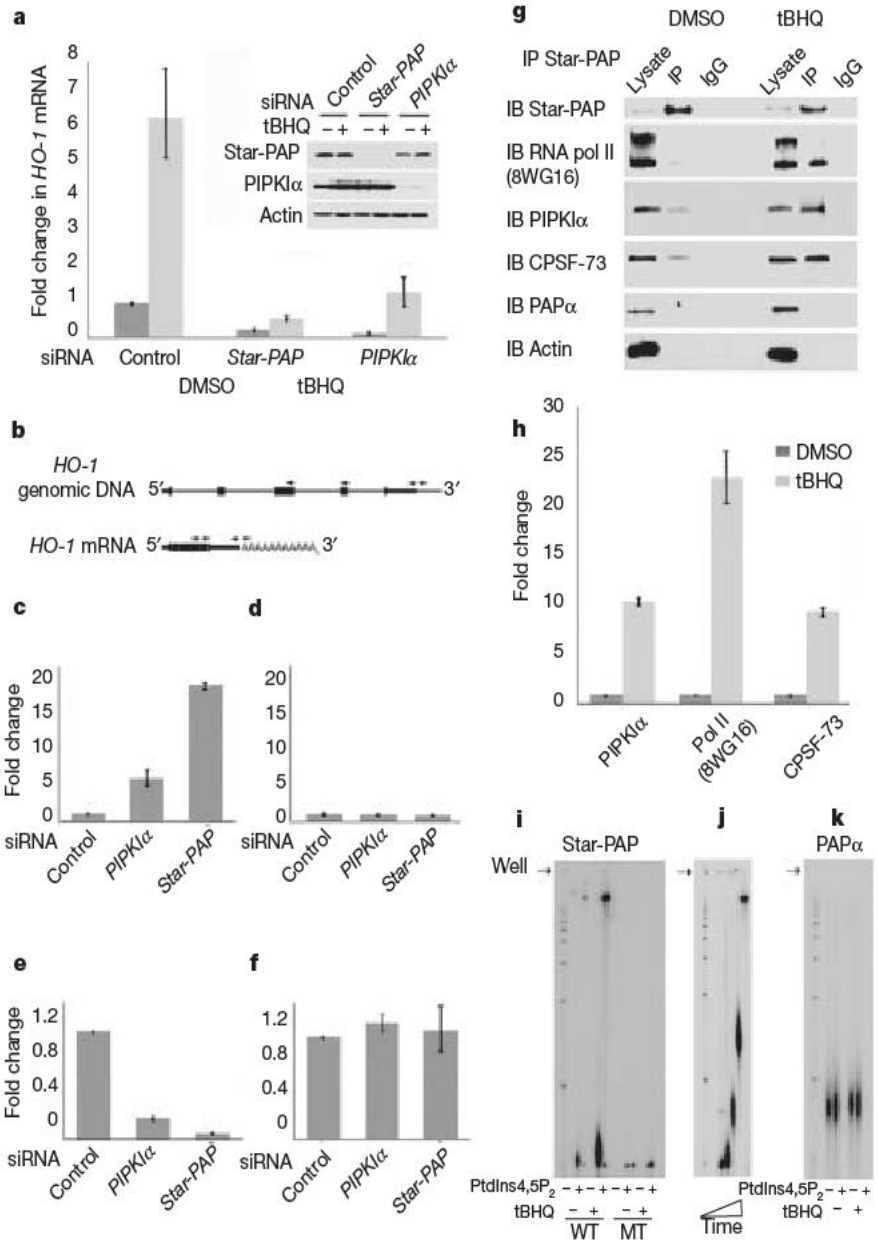

Figure 4 | Star-PAP and PIPKIα are required for efficient 3′ processing of *HO-1* mRNA by a mechanism that assembles a Star-PAP complex. **a**, qRT–PCR analysis of *HO-1* mRNA levels from HEK-293 cells transfected with *Star-PAP*, *PIPKIα* or control siRNA oligonucleotides and treated with 100 μM tBHQ ($n = 3$). DMSO, dimethylsulphoxide, vehicle control. **b**, Schematic diagram of primer set positioning used in *HO-1* mRNA cleavage analysis. **c**, **d**, Levels of uncleaved *HO-1* (**c**) and *GCLC* (**d**) mRNAs from HEK-293 cells transfected with control, *Star-PAP* or *PIPKIα* siRNA oligonucleotides. **e**, **f**, Results from **c** and **d**, respectively, normalized to total mRNA levels ($n = 3$). **g**, Immunoprecipitation (IP) of Star-PAP and detection of associated proteins from HEK-293 cells after treatment with 100 μM tBHQ. IB, immunoblot. **h**, Quantification of Star-PAP complex assembly from **g** ($n = 3$). **i**, PAP assay with affinity-purified Flag–Star-PAP (WT) or Flag–Star-PAP mutant (MT) from stably expressing HEK-293 cells subsequent to treatment with tBHQ and/or PtdIns4,5P_2. **j**, Time course subsequent to treatment with tBHQ in **i**, in the presence of PtdIns4,5P_2. **k**, Flag–PAPα activity after treatment with 100 μM tBHQ and/or the presence of PtdIns4,5P_2. All error bars represent s.e.m.

of its target mRNAs, revealing a unique function for nuclear phosphoinositides in the regulation of mRNA 3′ processing. The requirements for both Star-PAP and PIPKIα in the expression of *HO-1* mRNA provide a mechanistic link between a nuclear phosphoinositide signal transduction pathway and gene expression. Star-PAP is the first reported example of any non-canonical PAP, poly(U) polymerase (PUP)[16,28,29] or TUTase that is responsive towards phosphoinositides and assembles with the transcriptional and 3′-end-formation machinery in a signal-dependent fashion. Our model proposes (Supplementary Fig. 1) that not all nuclear pre-mRNAs require processing by PAPα. Instead, the identity and activity of the integrated PAP, PUP or TUTase varies between mRNAs in response to signalling cascades to regulate both mRNA stability and expression.

METHODS SUMMARY

Yeast two-hybrid screen. The yeast two-hybrid screen was performed by The Molecular Interaction Facility at the University of Wisconsin at Madison. Libraries screened were mouse embryonic, mouse B cell, human breast, human prostate, human placenta and mouse brain.

***In vitro* poly(A) polymerase assay.** PAP assays were performed as reported with RNA primers[13] and [α-^{32}P]ATP. An A_{15} RNA oligonucleotide was used as a substrate for the dose-dependent response of Star-PAP activity; for all other polyadenylation assays either L1 (ref. 11) or a 45-mer of the sequence $(UAGGGA)_5A_{15}$ designed to interact specifically with the Star-PAP RNA recognition motif [30] was used. The RNA product was extracted, precipitated with ethanol and dissolved in 2 × urea sample buffer and separated by PAGE in the presence of 6% urea. For the PIPn stimulation assay, the purified His-tagged Star-PAP was incubated for 10 min with PIPn micelles (Echelon Biosciences) on ice. Products were detected by a Storm 840 phosphorimager (Molecular Dynamics) and incorporation was quantified with NIH ImageJ software.

qRT–PCR and analysis of *HO-1* mRNA cleavage. Total RNA was purified from HEK-293 cells transfected with Star-PAP or PIPKIα-specific or control siRNA oligonucleotides with the RNeasy mini kit (Qiagen). For measurements of mRNA expression, RNA was reverse-transcribed with poly$(dT)_{20}$ primers and SuperScript III reverse transcriptase (Invitrogen). For assessment of mRNA cleavage, total RNA was treated with DNase I (Invitrogen) and then re-purified on RNeasy columns (Qiagen) before reverse transcription with random hexamer primers. The resulting complementary DNAs were used for qRT–PCR analysis with SYBR green detection chemistry on an ABI Prism 7000 sequence detection system (Applied Biosystems Inc.). Target mRNA levels were normalized to *GAPDH* levels. For measurement of mRNA, primers were designed to span intronic sequences to remove the possibility of DNA contamination. When this was not possible, RNA samples were treated with DNase I before RT–PCR.

Full Methods and any associated references are available in the online version of the paper at www.nature.com/nature.

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Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

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Author Contributions D.L.M. contributed to Figs 1a, d, f, 2, 3a, b, 4g–k and Supplementary Figs 1, 2, 4–7. M.L.G. contributed to Figs 1a, 3c, f, 4a–f and Supplementary Fig. 1. C.A.B. contributed to Fig. 3d, e, Supplementary Fig. 1 and Supplementary Tables 1 and 2. C.S. contributed to Fig. 1a, c, e and Supplementary Figs 3 and 6. P.W. and C.K. analysed the microarray data. R.A.A. directed the experimental approach and project. D.L.M., M.L.G., C.A.B. and R.A.A. analysed and interpreted the experiments, and conceptualized and wrote the paper.

Author Information The Star-PAP sequence is deposited in the NCBI Library under accession number NP_073741. The microarray data discussed in this publication have been deposited in NCBI's Gene Expression Omnibus (GEO, http://www.ncbi.nlm.nih.gov/geo/) and are accessible through GEO series accession number GSE9361. Reprints and permissions information is available at www.nature.com/reprints. Correspondence and requests for materials should be addressed to R.A.A. (raanders@wisc.edu).

LETTERS

Formation and branch migration of Holliday junctions mediated by eukaryotic recombinases

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Holliday junctions (HJs) are key intermediates in homologous recombination and are especially important for the production of crossover recombinants[1–4]. Bacterial RecA family proteins promote the formation and branch migration of HJs *in vitro* by catalysing a reciprocal DNA-strand exchange reaction between two duplex DNA molecules, one of which contains a single-stranded DNA region that is essential for initial nucleoprotein filament formation[5]. This activity has been reported only for prokaryotic RecA family recombinases[5], although eukaryotic homologues are also essential for HJ production *in vivo*[6,7]. Here we show that fission yeast (Rhp51) and human (hRad51) RecA homologues promote duplex–duplex DNA-strand exchange *in vitro*. As with RecA, a HJ is formed between the two duplex DNA molecules, and reciprocal strand exchange proceeds through branch migration of the HJ. In contrast to RecA, however, strand exchange mediated by eukaryotic recombinases proceeds in the 3′→5′ direction relative to the single-stranded DNA region of the substrate DNA. The opposite polarity of Rhp51 makes it especially suitable for the repair of DNA double-strand breaks, whose repair is initiated at the processed ends of breaks that have protruding 3′ termini[1,2].

ATP-dependent DNA-strand exchange is catalysed by recombinases that belong to the evolutionarily conserved RecA family[5,8,9]. These recombinases initiate strand exchange by forming a helical nucleoprotein filament on single-stranded (ss)DNA[5,8,10]. The filament aligns and pairs with a homologous double-stranded (ds)DNA, and strand exchange results when the complementary strand of the DNA duplex is transferred to the original ssDNA in the nucleoprotein filament. Because ssDNA regions exist as gaps in dsDNA or as processed ends at DNA double-strand breaks (DSBs), one likely mechanism for the formation of HJs is that strand exchange proceeds over a ssDNA–dsDNA junction. Only bacterial RecA protein has been shown to promote this type of so-called four-strand exchange *in vitro* (Supplementary Fig. 1). Although eukaryotic RecA homologues can promote DNA-strand exchange between ssDNA and dsDNA, this is restricted to D-loop formation and three-strand exchange (Supplementary Fig. 1)[5]. Because Rhp51, a fission yeast (*Schizosaccharomyces pombe*) Rad51 homologue, promotes the three-strand exchange reaction in a Swi5–Sfr1-dependent manner[11], we now examine whether Rhp51 promotes four-strand exchange.

The reaction is initiated by mixing a recombinase with a circular duplex DNA containing a 0.6-kilobase ssDNA region (gapped DNA (gDNA)) and its homologous linear duplex DNA (ldsDNA) (Fig. 1a). The ssDNA region of the gDNA pairs with its homologous region in ldsDNA, yielding a σ-structure, which is the first joint molecule (JM)

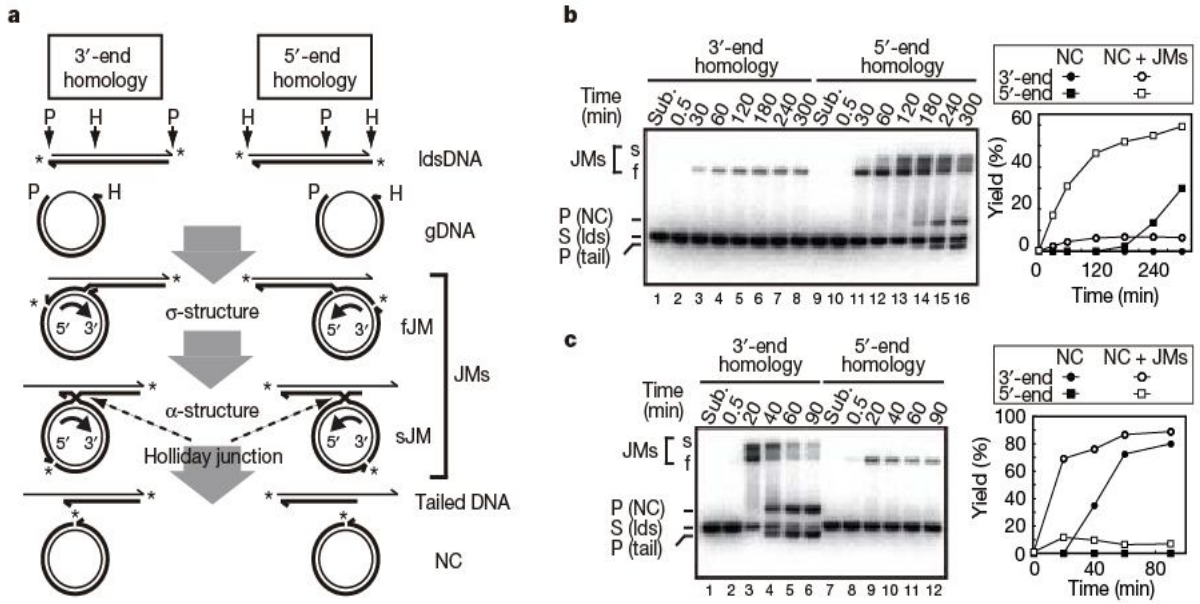

Figure 1 | Rhp51 promotes a DNA four-strand exchange reaction *in vitro*. **a**, Diagram of the four-strand exchange reaction. Asterisks designate the radiolabelled DNA ends. Half arrowheads indicate the 3′ ends. P and H designate the *Pst*I and *Hin*dIII recognition sites, respectively. **b**, **c**, Gel images showing the time-course analysis of the Rhp51-mediated (**b**) and RecA-mediated (**c**) four-strand exchange reaction. Sub, reactions were performed without protein components (**b**, 300-min incubation; **c**, 90-min incubation). Aliquots (6.5 μl) from total reactions (50 μl) were withdrawn at the indicated times. s, sJM; f, fJM.

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intermediate. Once strand exchange proceeds over the ssDNA–dsDNA junction of gDNA, an α-structure containing a HJ is produced; this is the second JM intermediate. Completion of DNA exchange results in nicked circular (NC) DNA and ldsDNA containing a ssDNA region (tailed DNA). The directionality of strand exchange can also be determined. The reaction with 5′→3′ polarity relative to the ssDNA region of gDNA occurs with *Pst*I-digested ldsDNA, whereas that with 3′→5′ polarity occurs with *Hin*dIII-digested ldsDNA. We term the former the 3′-end homology reaction and the latter the 5′-end homology reaction. It was previously established that RecA preferentially promotes strand exchange in the 5′→3′ direction relative to the ssDNA[12–14].

We monitored the time course of the four-strand exchange reaction promoted by Rhp51 (Fig. 1b). We also added the Swi5–Sfr1 complex, a Rhp51 mediator, and the ssDNA-binding protein replication protein A (RPA) to the reaction mixture because they greatly enhance Rhp51-mediated three-strand exchange[11]. These proteins alone did not mediate the four-strand exchange reaction (Supplementary Fig. 2). In the 5′-end homology reaction, two JM bands were detected. The faster-migrating JM band (fJM) was detected at an earlier time than the slower-migrating JM band (sJM). The intensity of the sJM band peaked at 180 min and then declined. Concomitantly, two bands corresponding to NC and tailed DNA became detectable (Fig. 1b, lanes 14–16). NC and tailed DNA were produced by means of JM formation, because the insertion of a heterologous sequence into ldsDNA distal to the site of initiation of strand exchange prevented the production of NC and tailed DNA but not the formation of JMs (Supplementary Fig. 3a, b). fJM became detectable at about the same time in the 3′-end homology reaction (Fig. 1b, lanes 2–8). However, little sJM formation was detectable and no NC or tailed DNA products were observed in the 3′-end homology reaction. Similar results were obtained with another gapped DNA containing a ssDNA gap at a different region, suggesting that Rhp51 activity does not require a specific type of gDNA construct (Supplementary Fig. 4). As a positive control, we performed the RecA-mediated reaction (Fig. 1c). In contrast to the Rhp51-mediated reaction, sJM, NC and tailed DNA were produced only in the 3′-end homology reaction with RecA and the reaction was much faster than that of Rhp51. These results agree very well with those of previous studies on RecA[12–14]. These results clearly indicate that Rhp51, in contrast with RecA, mediates the four-strand exchange reaction 3′→5′ relative to the ssDNA gap.

To determine whether the JMs resulting from Rhp51-mediated strand exchange contained Holliday structures, we used *Escherichia coli* RuvC as a probe. RuvC is an endonuclease that specifically introduces symmetrical nicks at junctions of Holliday intermediates[15]. As illustrated in Fig. 2a, two different products are generated, depending on the direction of RuvC digestion. One product is a nicked linear dsDNA that is twice as long as the original ldsDNA (a linear dimer). The other products are a nicked NC and a nicked tailed DNA. Because they are also the final products of the four-strand exchange reaction, the production of the linear dimer can be used to indicate HJ production[16]. Both the 3′-end homology reaction mediated by RecA and the 5′-end homology reaction mediated by Rhp51 produced linear dimer molecules on the addition of RuvC (Fig. 2b). A similar result was observed in the four-strand exchange reaction with the use of ldsDNA containing a heterologous insertion sequence as a substrate (Supplementary Fig. 3c). α-shaped DNA molecules containing a HJ were also observed by electron microscopy in the Rhp51-mediated 5′-end homology reaction (Fig. 2c). These results clearly indicate that the JM formed in the 5′-end homology reaction mediated by Rhp51 contains a HJ.

As in the three-strand exchange reaction[11], a substoichiometric amount of Swi5–Sfr1 stimulated the Rhp51-mediated four-strand exchange reaction (Supplementary Fig. 5a). However, the dependence on Swi5–Sfr1 was less strict than in the three-strand exchange[11,17]. In the absence of Swi5–Sfr1, the amounts of the intermediate and final products were decreased by about one-half (Fig. 3a and Supplementary Figs 2 and 5a), whereas there were no detectable products in the three-strand exchange reaction in the absence of Swi5–Sfr1 (data not shown; see also ref. 11). The timings of the appearances of JMs and NC were almost the same in the presence and in the absence of Swi5–Sfr1 in the four-strand reaction (Fig. 3a), indicating that Swi5–Sfr1 stimulates JM formation but does not affect the velocity of branch migration by Rhp51. RPA or bacterial ssDNA-binding protein (SSB) stimulated the reaction almost equally well (Supplementary Figs 2b and 5b). Although the addition of either NaCl or KCl stimulated the reaction, the production of NC and tailed DNA was clearly more robust with NaCl than with KCl (compare Supplementary Fig. 6a with Supplementary Fig. 6b). The nature of the salt cation made little difference to the three-strand exchange reaction (data not shown). The effective range of magnesium concentrations was very narrow and the optimal concentration was about 10 mM, which was slightly higher than that for the three-strand exchange reaction (Supplementary Figs 6c and 7). These features stress the marked differences in the requirements for the two reactions and show that the four-strand exchange requires stricter reaction conditions with narrower optimal ranges than the three-strand exchange reaction.

As expected, no final products were detected in the absence of ATP or in the presence of ADP (Fig. 3b, lanes 7 and 10). Omission of the ATP regeneration system did not significantly affect the reaction

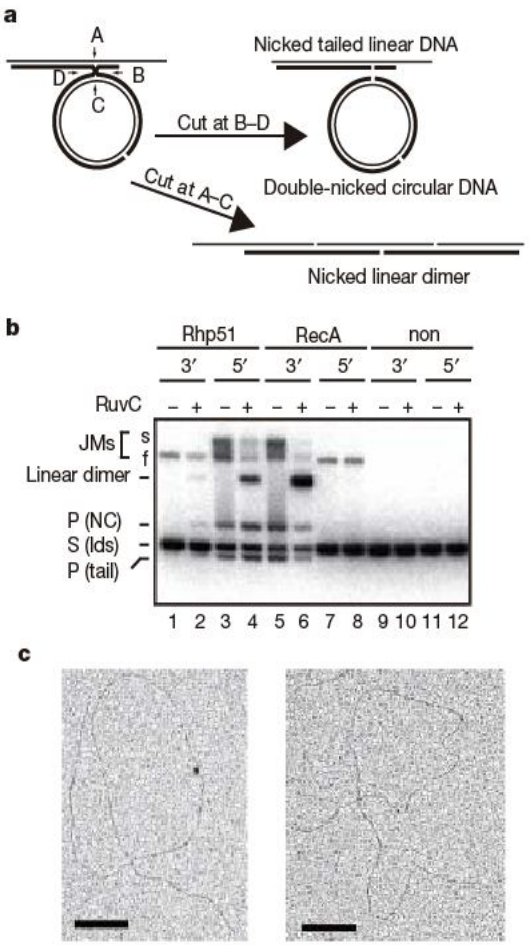

Figure 2 | Formation of HJs in the Rhp51-mediated four-strand exchange reaction. **a**, RuvC generates products of two types from an α-structure sJM containing Holliday structures. **b**, Gel image of the RuvC-digested products. Plus and minus signs indicate the addition of RuvC or buffer alone, respectively. The lanes labelled 3′ and 5′ represent a 3′-homology and 5′-end homology reaction, respectively. Non, reactions performed without protein components. **c**, Analysis of HJ formation by electron microscopy. Left: Rhp51-mediated 5′-end homology reaction (180-min incubation). Right: RecA-mediated 3′-end homology reaction (30-min incubation). Scale bar, 200 nm.

(Fig. 3b, lane 9). Interestingly, ATP-γS did not support the reaction at a detectable level, whereas β-γ-imidoadenosine 5′-phosphate (AMP-PNP) supported a substantial amount of JM formation (Fig. 3b, lanes 11 and 12). However, AMP-PNP supported only the production of fJM (but not sJM, NC or tailed DNA). Because the production of the final branch migration products, NC and tailed DNA, correlated with the appearance of sJM (Fig. 3b, lanes 8, 9 and 11), we speculate that sJM was the only stable intermediate with a HJ (α-structure) and that the fJM band consisted mostly of an intermediate with a σ-structure formed in an early phase of the four-strand reaction (see Fig. 1a).

We suggested that AMP-PNP cannot support strand exchange over the ssDNA–dsDNA junction of gDNA because ATP hydrolysis is required for reciprocal strand exchange between two duplex molecules. To test this hypothesis, we used RuvC to probe the fJM species produced with AMP-PNP to see whether it contained a HJ. As expected, no fJM cleavage products were detected in the presence of RuvC for either the 5′-homology or 3′-homology reactions (Fig. 3c). Direct observation of the DNA molecules by electron microscopy also indicated that AMP-PNP could support the production of σ-structures but not of α-structures (Supplementary Fig. 8). ATP hydrolysis was required for the branch migration of HJs, because the addition of AMP-PNP caused the accumulation of sJMs and retarded the formation of the final NC and tailed DNA species in an ATP-initiated reaction (Fig. 3d). Taken together, our findings demonstrate that ATP (AMP-PNP) binding is sufficient for the fJM (σ-structure) formation that results from three-strand exchange between the short ssDNA in gDNA and ldsDNA, but that ATP hydrolysis is critical for the formation and branch migration of the HJ in the four-strand exchange reaction. The same conclusion for ATP hydrolysis was drawn from the RecA-mediated four-strand exchange reaction[18,19].

It is not clear whether the catalytic ability and the polarity of the four-strand exchange reaction is a common feature of eukaryotic recombinases. Previous reports demonstrated that the polarity of the three-strand exchange reaction mediated by Rad51 recombinases from budding yeast and human cells was not very strict[20–26]. This is in marked contrast to the strict polarity of Rhp51 in the four-strand exchange reaction demonstrated here. To examine whether the polarity of Rhp51-mediated strand exchange is unique to the four-strand exchange reaction and whether Rhp51 shows the same non-strict polarity for the three-strand exchange as other eukaryotic recombinases, we analysed the three-strand exchange reactions. The results indicated that Rhp51 can promote the three-strand exchange in both directions with a slight preference for the 3′→5′ polarity and that the polarity is affected by the end structure of ldsDNA (Supplementary Fig. 9b, lanes 2–13). This is in contrast with the polarity of RecA (Supplementary Fig. 9b, lanes 15–26)[27–29]. All of these results show that Rhp51 has similar properties to those of other eukaryotic Rad51 recombinases reported previously[20–26], indicating that fission yeast Rhp51 is not an exceptionally 'rare' recombinase among Rad51-type recombinases in eukaryotes.

To address the issue more directly, we tested whether hRad51 could also mediate the four-strand exchange reaction. As shown in Fig. 4a, fJM was observed after 2 h of incubation of hRad51 in the 5′-end homology reaction. sJM was also observed at 8 h, whereas NC and tailed DNA products were not detected at this time point (Fig. 4a, lane 11). After 12 h, the final products were detectable and their amounts increased in a time-dependent manner thereafter (Fig. 4a, lanes 14–16). In the 3′-end homology reaction, no products were detected within 8 h (Fig. 4a, lanes 2–5). This indicates that hRad51 mediates the four-strand exchange reaction with the same polarity as Rhp51, although its efficiency was comparatively very low.

We next examined whether the JMs formed by hRad51 contained HJs. Because RuvC-cleavage activity is impeded by the high salt conditions required for the hRad51-mediated strand exchange, the reaction mixtures were deproteinated and exchanged with low-salt buffer before examining RuvC cleavage of the JMs formed by hRad51. As shown in Fig. 4b and Supplementary Fig. 10, the JMs formed by hRad51, like those formed by RecA, were cleaved by RuvC, resulting in the generation of a linear dimer product. These results clearly

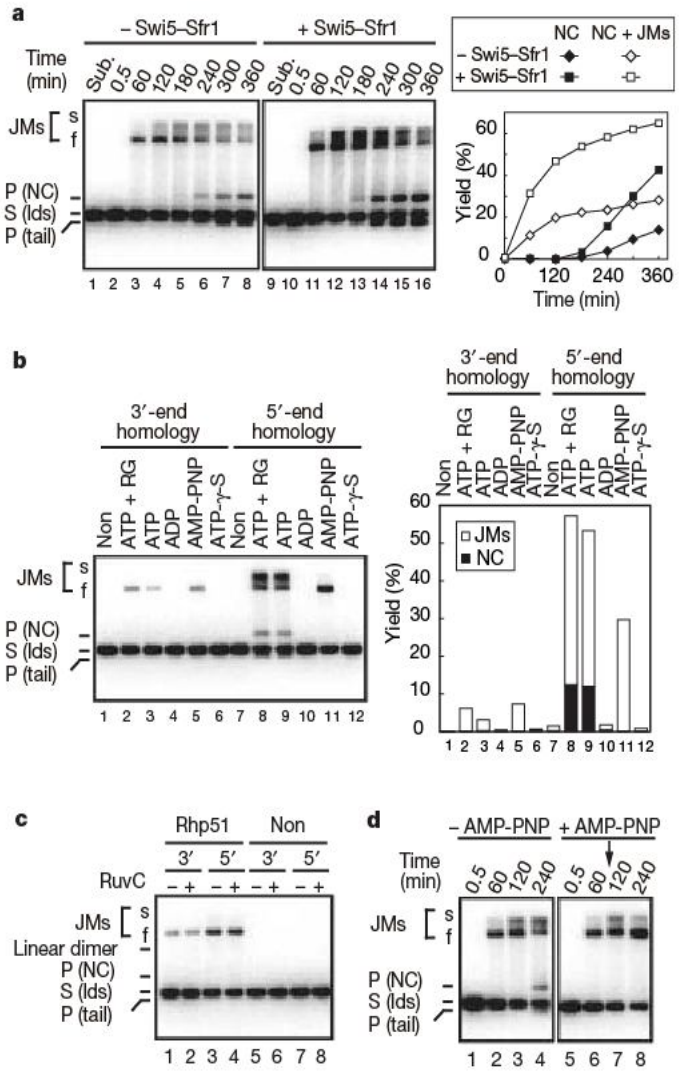

Figure 3 | Effects of Swi5–Sfr1 and adenine nucleotides. **a**, Time-course analysis of the 5′-end homology reaction with and without Swi5–Sfr1. **b**, Effects of the indicated adenosine derivatives (2 mM each). All reactions were performed without an ATP regeneration system with the exception of lanes 2 and 8 (indicated by +RG). **c**, fJMs generated from AMP-PNP-mediated reactions could not be cleaved by RuvC. **d**, Reactions initiated in the presence of 1 mM ATP (plus an ATP regeneration system). AMP-PNP was added at the 60-min time point at a final concentration of 1 mM.

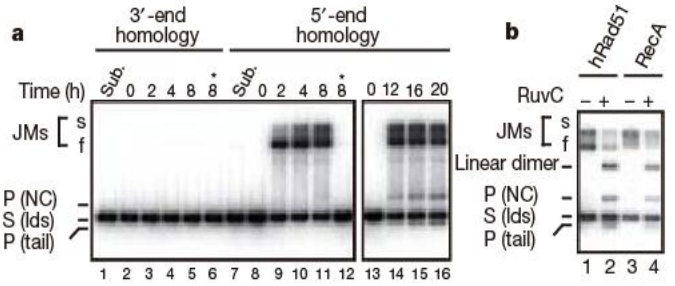

Figure 4 | hRad51 promotes the DNA four-strand exchange reaction *in vitro*. **a**, Time-course analysis of the hRad51-mediated reaction. The reactions in lanes 6 and 12 were performed without ATP for 8 h. At 20 h, about 8% of the input ldsDNA was converted to NC products (and about 60% to JMs). **b**, RuvC-cleavage of JMs formed by the hRad51-mediated four-strand reaction. Lanes 1 and 2, 5′-end homology reaction mediated by hRad51 (at 6-h time point); lanes 3 and 4, 3′-end homology reaction mediated by RecA (at 30-min time point).

showed that hRad51 can promote the formation and branch migration of HJs. The catalytic activity with the opposite polarity to that of RecA is therefore an intrinsic feature of eukaryotic recombinases.

To promote the four-strand exchange reaction, the recombinase filament must extend over the ssDNA–dsDNA junction. In addition, the reciprocal exchange between two duplex DNA molecules that accompanies consecutive Rhp51 assembly over the ssDNA–dsDNA junction requires ATP hydrolysis (Fig. 3b–d). This implicates ATP hydrolysis in the determination of polarity. The observed polarity is quite compatible with recombination repair models, including the DNA double-strand break repair model[1,2]. In that model, the 3′ overhang invades the homologous duplex DNA, leading to D-loop formation. Subsequently, strand exchange proceeds in the 3′→5′ direction to form a HJ (Supplementary Fig. 1). The 3′→5′ polarity of Rhp51 and hRad51 discovered in this study is much more suitable for this purpose than the 5′→3′ polarity of RecA. The very low activity observed implies the existence *in vivo* of accessory factors that assist these recombinases in the four-strand exchange and/or promotion of branch migration, as occurs with the bacterial RuvAB branch migration motor protein[15]. Rad54 is an example of one eukaryotic factor that could have such a function *in vivo*[30]. Our findings provide a new approach to unravelling the precise mechanisms of Rad51-mediated strand exchange and homologous recombination in eukaryotes.

METHODS SUMMARY

Strand exchange reaction. In the standard reaction, Rhp51 and Swi5–Sfr1 were premixed, and then gDNA (for the four-strand exchange reaction) or circular ssDNA (for the three-strand exchange reaction) was added. RPA was then added to the mixture before the reaction was initiated with ldsDNA. The products were analysed by agarose-gel electrophoresis. RecA-mediated and hRad51-mediated reactions were performed essentially as for Rhp51 except that SSB was used instead of RPA, and Swi5–Sfr1 was not added to the reactions.

RuvC nuclease test. Four-strand exchange reactions were initiated as above before the addition of RuvC to the reaction mixture. The products were analysed by agarose-gel electrophoresis. For hRad51, the strand exchange reactions were deproteinated and desalted before treatment with RuvC.

Electron microscopy. Samples were crosslinked with psoralen before deproteinization. The spreading of the sample DNA was performed by the droplet method, using cytochrome *c* as a carrier protein. The DNA film was picked up on carbon-coated grids, stained with 2% uranyl acetate, and examined in an electron microscope.

Full Methods and any associated references are available in the online version of the paper at www.nature.com/nature.

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Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

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Author Contributions Y.M., Y.K. and H.I. designed the experiments. Y.M. and Y.K. performed the experiments. K.M. performed the electron microscopy analysis. Y.M. and H.I. wrote the manuscript.

Author Information Reprints and permissions information is available at www.nature.com/reprints. Correspondence and requests for materials should be addressed to H.I. (iwasaki@tsurumi.yokohama-cu.ac.jp).

CORRIGENDUM

doi:10.1038/nature06807

Depth of a strong jovian jet from a planetary-scale disturbance driven by storms

A. Sánchez-Lavega, G. S. Orton, R. Hueso, E. García-Melendo, S. Pérez-Hoyos, A. Simon-Miller, J. F. Rojas, J. M. Gómez, P. Yanamandra-Fisher, L. Fletcher, J. Joels, J. Kemerer, J. Hora, E. Karkoschka, I. de Pater, M. H. Wong, P. S. Marcus, N. Pinilla-Alonso, F. Carvalho, C. Go, D. Parker, M. Salway, M. Valimberti, A. Wesley & Z. Pujic

Nature 451, 437–440 (2008)

In Fig. 3a, the descriptions of the continuous and dotted curves were inadvertently swapped. The continuous line corresponds to the modified synthetic thermal profile (storms reaching the 60 mbar level). The dotted line corresponds to the Cassini CIRS thermal profile (storms reaching the 160 mbar level).

ERRATUM

doi:10.1038/nature06729

Roquin represses autoimmunity by limiting inducible T-cell co-stimulator messenger RNA

Di Yu, Andy Hee-Meng Tan, Xin Hu, Vicki Athanasopoulos, Nicholas Simpson, Diego G. Silva, Andreas Hutloff, Keith M. Giles, Peter J. Leedman, Kong Peng Lam, Christopher C. Goodnow & Carola G. Vinuesa

Nature 450, 299–303 (2007)

In this Letter, some axis labels in Figs 3 and 4 were inadvertently mislabelled. In Fig 3d, the labels on the *x* axes of the three graphs should read 'Hi Low Nil' instead of 'Hi Low Ni'. The *x* axes of the bar graphs in Fig. 4a (right and left panels) and Fig. 4b (right panel) should read 'Hi Low Nil' instead of 'Hi Low Hi'.

CORRIGENDUM

doi:10.1038/nature06728

The nonlinear Fano effect

M. Kroner, A. O. Govorov, S. Remi, B. Biedermann, S. Seidl, A. Badolato, P. M. Petroff, W. Zhang, R. Barbour, B. D. Gerardot, R. J. Warburton & K. Karrai

Nature 451, 311–314 (2008)

The experiment measures the differential laser transmission through the quantum dot between the on- and off-exciton resonance condition. As a result, the origin in Fig. 2a–h corresponds to the zero of the measured differential transmission and does not exclude the existence of constant background absorption. It is therefore important to note that the undershoot in the Fano spectra does not correspond to a negative absorption (that is, an optical gain), but is consistent with the continuum broadband background absorption, as analysed in our observation of the nonlinear Fano effect. Similarly, the theoretical graphs in Fig. 2i–n are also given for the differential transmission.

CORRIGENDUM

doi:10.1038/nature06779

Systems biology approaches identify ATF3 as a negative regulator of Toll-like receptor 4

Mark Gilchrist, Vesteinn Thorsson, Bin Li, Alistair G. Rust, Martin Korb, Jared C. Roach[1], Kathleen Kennedy, Tsonwin Hai, Hamid Bolouri & Alan Aderem

[1]Institute for Systems Biology, Seattle, Washington 98103, USA.

Nature 441, 173–178 (2006)

In this Article, Jared C. Roach was inadvertently omitted from the list of authors. He was responsible for designing the immune-specific array for ChIP-to-chip analysis. J.C.R. received support from the National Institute of Allergy and Infectious Diseases, National Institutes of Health.

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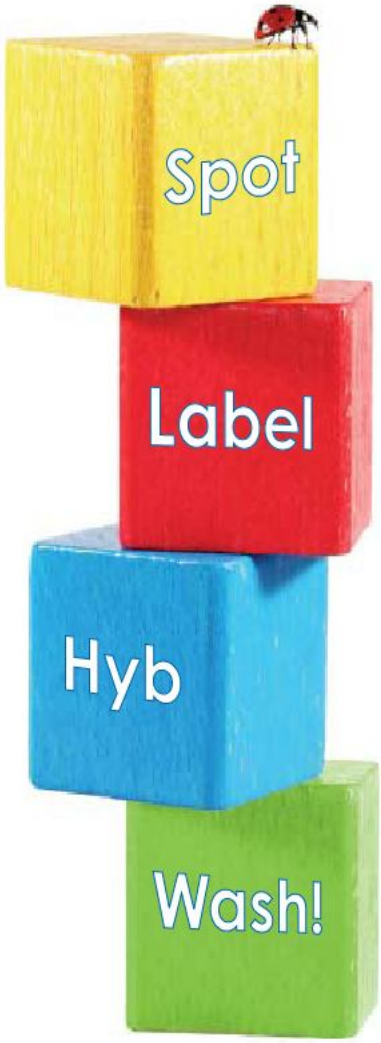

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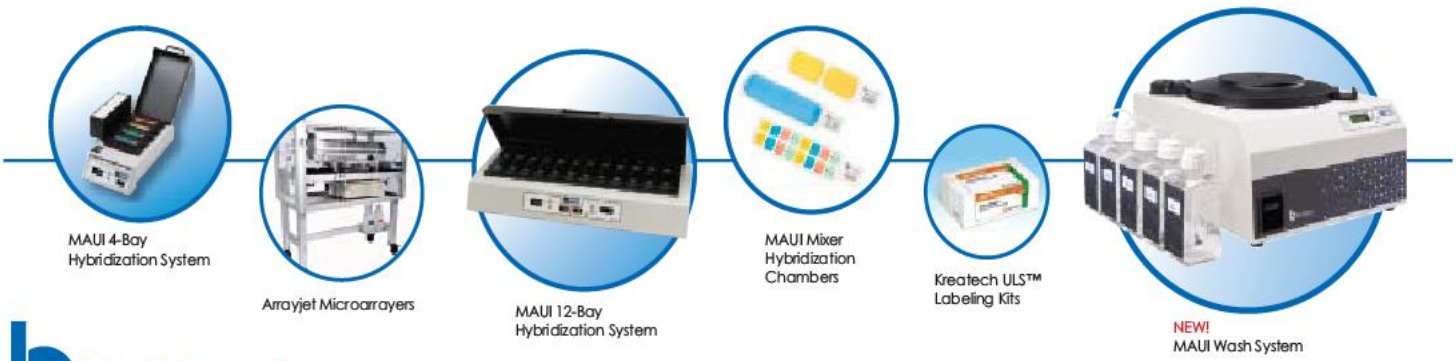

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Illumina has introduced two ground-breaking new products for DNA analysis: the **Infinium HD Human1M-Duo** (two samples/chip) and the **Human610-Quad** (four samples/chip), featuring up to 2.3 million Single Nucleotide Polymorphisms (SNPs) per BeadChip. The Infinium HD product doubles sample throughput and reduces DNA input requirements by as much as 70 percent. Additionally, the Infinium HD products offer enhanced signal discrimination and a new SNP calling algorithm. Both arrays on the Human1M-Duo BeadChip contain markers for more than one million diverse genetic variants, all of which can be used for both whole-genome genotyping and copy number variation (CNV) analysis. Built upon the content of Illumina's broadly adopted HumanHap550 BeadChip, the Human610-Quad BeadChip has 550,000 SNPs plus an additional 60,000 genetic markers per sample.

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Kits and reagents

Agilent Technologies and **Kreatech Biotechnology** have launched a labelling kit that enables oligo microarray comparative genomic hybridization (CGH) analysis of

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DNA from formalin-fixed paraffin-embedded (FFPE) tissue samples. The method is based on Kreatech's Universal Linkage System (ULS) technology, a non-enzymatic direct labelling methodology that has been optimized for Agilent oligo CGH microarrays. This new labelling technology also features a simple, single-tube protocol, enabling a reduced cost per experiment. "One of the key advantages of our ULS labelling technology is that it eliminates the bias often found with enzymatic labelling methods, so one can label DNA of any fragment size," said Dimitri Pappaioannou, Senior Product Manager at Kreatech. There are an estimated 400 million FFPE-preserved samples in tissue banks worldwide, and the DNA in these samples has been considered too degraded to use in microarray analysis techniques such as aCGH, a powerful method for studying DNA copy number variation in cancers and genetic disorders.

Eppendorf's DualChip GMO kit is the first fully validated microarray-based technology for the screening of genetically modified organisms (GMO) in a single test. The selection of the detectable organisms can be extended according to the growing number of approved and unapproved GMOs which have to be screened in the near future. The technology allows the parallel screening for multiple GMOs in food and feed. GMO-specific sequence information is hereby amplified by PCR, and the material is subsequently analyzed on microarrays.

The **TrueLabeling-PicoAMP Kit** from **SuperArray** is designed to perform two rounds of amplification and labeling of an antisense or complimentary RNA target for hybridization to high-density genome microarrays like the Agilent Gene Expression DNA Microarrays or the pathway-focused Oligo GEArray® from SuperArray Bioscience. Starting from as little as 20 cells or 50 pg of total RNA it is possible to generate enough labeled cRNA for microarray hybridization. The two rounds of amplification improve sensitivity from these samples, providing the same positive call rate as larger amounts of cells or RNA using standard one-round methods. The linear RNA amplification and labelling procedure of TrueLabeling-PicoAMP™ also maintains the original gene expression profile.

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Array analysis and accessories

Researchers at **SuperArray Bioscience** have evaluated the **Oligo GEArrays** using **Syngene's G:BOX Chemi HR16** and **G:BOX Chemi XT16** imaging systems. They found using a G:BOX Chemi HR16, they could image eight arrays simultaneously and with the G:BOX Chemi XT16, 32 arrays at the same time. They also showed both systems had excellent dynamic range and their cooled cameras allowed the long exposure times of 20 minutes often required to get maximum signal, with low background noise on the resulting images. Dr Ray Blanchard, Senior Scientist at SuperArray Bioscience explained: "Many researchers use X-ray film, followed by scanning and densitometry to analyze chemiluminescent microarrays, but by doing this, they can lose around 80 percent of the information just in the handling. Using the right CCD based system, scientists can capture much more data, producing quantitative analysis and, as a result, reliable gene expression measurements."

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"Using the right CCD based system, scientists can capture much more data, producing quantitative analysis and, as a result, reliable gene expression measurements"

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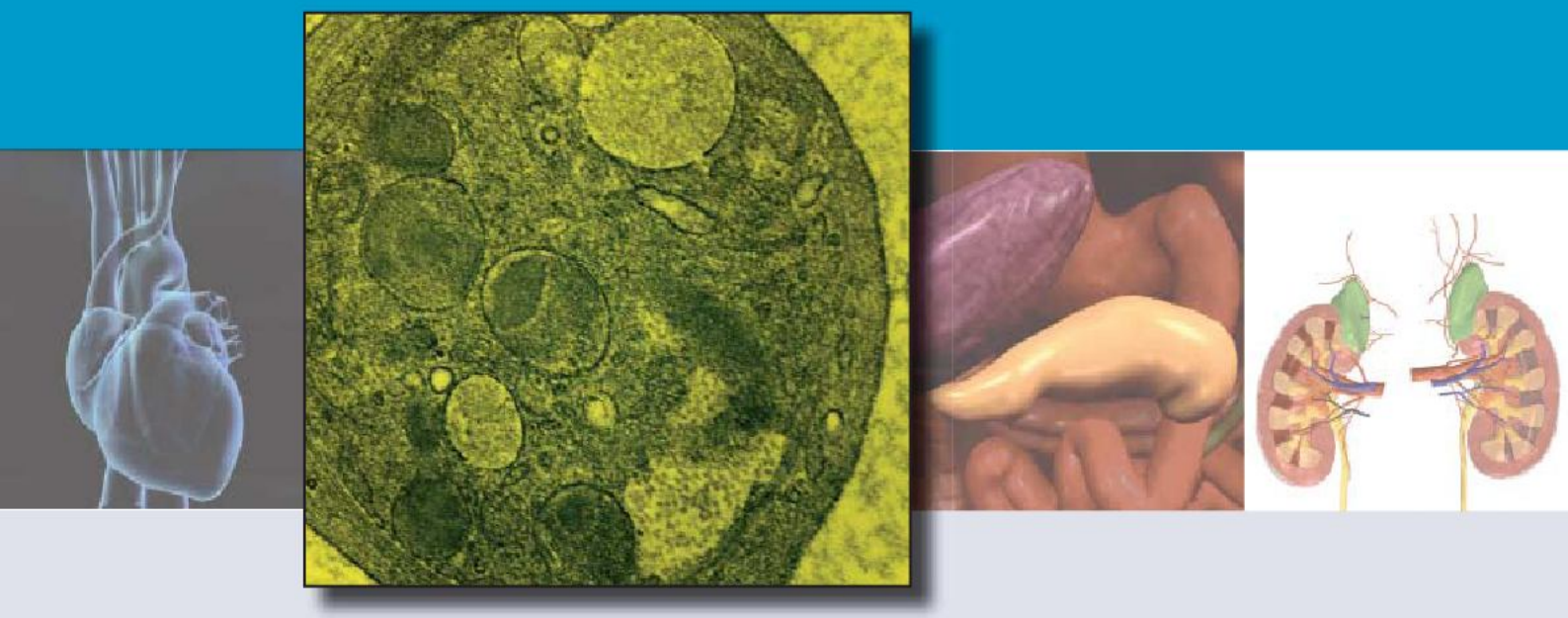

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This research division is focused on molecular and cellular function discovery of experimentally yet uncharacterized genes. It comprises a protein sequence-analytic team, a biochemical laboratory for function verification and a group developing the ANNOTATOR software environment for efficient protein sequence-analytic workflows. Based primarily on protein sequence analysis and the analysis of other sequence-associated data, the various aspects of molecular and cellular function (enzymatic activities, posttranslational modifications, 3D structures, translocation signals, pathway relationships, etc.) are predicted. These hypotheses are either followed up by experimental collaborators or they are validated in our own protein biochemical laboratory.

Candidates for the biochemical laboratory (headed by Dr. Manfred Koranda) are expected to have a PhD in protein biochemistry or in a closely related field and are expected to follow up biological consequences of newly predicted protein functions experimentally and, thus, get involved in the discovery of new biomolecular mechanisms.

Candidates for the sequence-analytic team (headed by Dr. Sebastian Maurer-Stroh and Dr. Frank Eisenhaber) are expected to move into the area of computational analysis of biomolecular data with the goal of gene/protein function prediction and a system-level understanding of the role of genes/proteins in pathways and networks. You will bring additional biological expertise into a collaborative, multidisciplinary team of life scientists, physicists, chemists and computer specialists. A PhD in Biochemistry, Molecular Biology, Computational Biology, or equivalent would be ideal. Candidates should preferably have simple programming skills (such as C/C++, Java, Perl, etc.), be familiar with several basic programs for sequence analysis (e.g. BLAST) and have an excellent background in Molecular Biology and Biochemistry. Any wet-lab experimental experience would be a positive asset.

Genome and Gene Expression Data Analysis Division

This research division attempts to understand transcriptional regulation and its relevance for phenotypic properties. The analysis of expression profiles and chromatin immuno-precipitation data for DNA-binding proteins can uncover transcriptional networks and it represents a step into systems biology. Gene expression signatures have considerable potential for diagnostic tasks including clinical applications.

Candidates for the group of Dr. Vladimir Kuznetsov will be involved in development of the research program in computational genomics and microarray data analyses and integrative bioinformatics. You will develop and apply computational, bioinformatics and statistical approaches to analyze complex large-scale data sets provided by modern sequence, structure and expression technologies. Your research is aimed at understanding the molecular basis of gene expression control, genome complexity and networks in normal and abnormal (e.g. cancerous) cells mediated by regulatory molecules including ncRNA and transcription factors. Candidates should have a PhD in Computational Biology, Systems Biology, Maths/Statistics or Biophysics. Strong programming skills (C++, BioPerl, R, Java, etc.) are required. You should possess good understanding of fundamentals of gene structure, expression and regulation, molecular evolution. Familiarity with RNA secondary structure analysis, ncRNA pathways, computational methods for large-scale genomic analysis, databases and graph theory methods is preferable.

Please submit your detailed resume with one-two page statement of research accomplishment and goals (if applicable) via email to **recruit@bii.a-star.edu.sg** or send to **The HR Department, 30 Biopolis Street, #07-01, Matrix, Singapore 138671**.

JP125436R

naturejobs

It was only a matter of time before scientists began to use the new 'friend finding' websites such as Friendster and MySpace as a way to establish collaborations. Such networking tools have the potential to change the way science is done: rather than swapping photos and music tips, researchers can discuss protocols and exchange assays. But there is a potential pitfall: the avid networker could end up with too many connections. Fewer good connections may be preferable to many mediocre ones.

The much heralded age of Web 2.0, with its avatars, virtual worlds and 'second lives', has made connecting with others across the globe easier than ever before. A researcher's links among potential colleagues and collaborators can proliferate as much as he or she likes. But time is precious, and making sure those connections contribute to a productive lab or field project might become an issue. Every technology has a downside. Quality, especially in the world of collaborative science, must trump quantity.

But the benefits are clearly outstanding. This week's feature (see page 1024) explores some of the uses — forming online groups to reach across disciplines, connecting clinicians with basic researchers, or sharing workflows to undertake statistical analyses of large data sets. Researchers can boost productivity and technical acumen.

Recognizing the caveats, I am pleased to announce that *Naturejobs* is launching a forum on Nature Network (http://network.nature.com/group/naturejobs) to offer discussion and advice on a range of issues related to science careers. Moderated by former *Naturejobs* editor Paul Smaglik, and with contributions from specialists in science careers, we will hold discussions that will, for example, help scientists who want to switch to a nontraditional career, explore salary or benefits issues, respond to *Naturejobs* content or enquire about science opportunities in a particular region. This virtual meeting ground will offer sage advice and tips from experts and those undertaking career decisions alike. Please join us — no avatars required.

Gene Russo, acting editor of *Naturejobs*

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JOBS OF THE WEEK

Green Chemistry Opportunities for talented Scientist
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Redwood, CA (USA)
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Phd program in Molecular Life sciences
Biozentrum-University of Basel
Basel (Switzerland)
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Senior Postdoctoral Fellow
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Research Faculty Positions
Department of Ophthalmology and Visual Sciences - University of Illinois at Chicago
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Professorship in Microbial Molecular Pathology
Trinity College Dublin
Dublin (Ireland)
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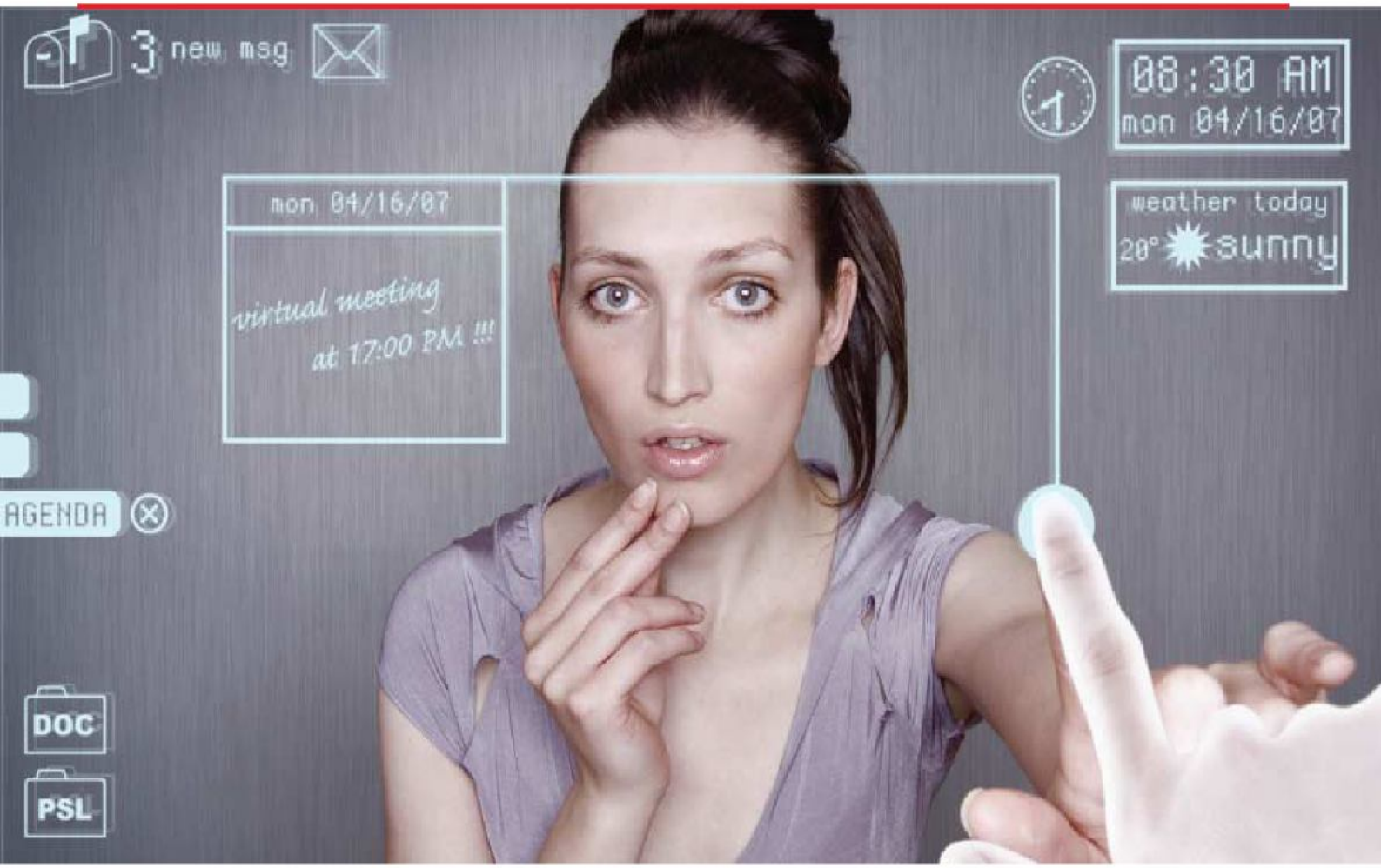

SIE PRODUCTIONS/ZEFA/CORBIS

The new networking nexus

A crop of websites is making networking among scientists easier than ever. **Virginia Gewin** logs in.

Compared with crafting computational expertise or sharpening gene-splicing skills, networking is one talent many scientists are slow to hone. Luckily, a crop of new websites is encouraging even the most reclusive researchers to rendezvous with colleagues without leaving the lab.

The success of social-networking websites such as MySpace, Facebook and LinkedIn shows the power of the Internet not only to cultivate, but to capitalize on, friendships. Although online networks may seem impersonal, they can do something for scientists that a handshake cannot: highlight common research interests without leaving the comfort of your desk. Say goodbye to name tags and awkward introductions — say hello to profiles and blogs. In the search for jobs, mentors, collaborators or data, these cyber-social mixers are revealing new ways to gain career advice, create collaborations and share resources.

Social scientists

To meet the constant demand for career advice, some scientific associations have harnessed existing sites to provide a networking option. For example, the American Association for the Advancement of Science (AAAS) and the American Institute of Biological Sciences (AIBS) have created networking groups on both Facebook and LinkedIn. There, members search for jobs, seek advice and discuss funding opportunities. Unfortunately, scientific specialities can easily get lost on social networking sites designed to amass large numbers of connections.

A growing number of websites, including Nature Network (a product of the Nature Publishing Group, the parent company of *Nature*) and Chemical Forums are coming online to meet more specific needs. Although these sites reach out to a broad spectrum of disciplines, scientists can create more focused forums, groups or blogs to spark more specialized discussions. Some of Nature Network's most popular forums are devoted to evolution and brain physiology. Chemical Forums enables its 7,000 chemists to segregate into everything from physical chemistry to chemical engineering. There's even a Citizen Chemist forum to exchange useful chemistry experiments or download chemistry games.

Scientists with common interests can connect across long distances and disparate scientific cultures. Although Nature Network has portals for London- and Boston-based researchers to find one another, the global forum is the most active. Nature Network editor Corie Lok says quite a few users from the developing world — most notably India, South America and the Middle East — use the site to connect with international colleagues. "For some segments of the scientific world that may feel isolated, online networking sites are a great place to connect," she says.

For example, government research job postings in Italy are hard to find, often posted only in Italian on specific newsletters or websites. Massimo Pinto, a radiology postdoc at the ISS, Italy's higher institute of health, decided to create a portal on Nature Network to translate, publish and so promote these jobs. He wants to encourage transparency and intellectual exchange

between Italy and other countries, traits he admired while doing his graduate work at the Gray Cancer Institute in Oxford, UK. He hopes to see networks create ways to check where people are, so scientists at a conference in Boston can see which of their contacts happen to be nearby for a face-to-face encounter.

With increased funding for cross-disciplinary science, many networks are experimenting with ways to help members collaborate. Chemical Forums founder and moderator Mitch André Garcia, a PhD student at the University of California, Berkeley, set up the Chemmunity website to facilitate a global collaboration to solve a chemical mystery — why hexaiodobenzene changes colour on introduction of liquid nitrogen, only to return to its original colour at room temperature. Garcia hopes it will encourage chemists to work on research outside their niche.

Interestingly, says Lok, interdisciplinary scientists have a strong presence on Nature Network. One computational biologist she spoke to suggested that might be due to the fact that these newer fields don't have established networking channels. As well, computational researchers log serious computer time.

K. KOTT/DIGITAL LIBRARY FEDERATION

"I developed the forum as a way for people to communicate outside the bounds of affiliations."
— Peter Brantley

Building a critical mass

Even computer-savvy investigators can find it difficult to kindle an active community. Peter Brantley, executive director of the Digital Library Federation in Washington DC, created a Datanet group on Nature Network to publicize a National Science Foundation (NSF) call for proposals to establish cyberinfrastructure centres of excellence internationally. "I developed the forum as a way for people to communicate outside the bounds of institutional affiliations," he says, but adds that it has been hard to achieve a critical mass of participation.

Another challenge is managing this type of site to ensure that the communication is protected, yet flexible enough to let users discuss sensitive topics. This is a concern for medical professionals, who have been using networks to discuss cases and get advice for more than a decade. For example, the non-profit Doctors.net.uk has firmly planted itself as a must in UK circles. Sermo and Within3 are two new US-based incarnations of the same idea, but for profit. Although Sermo sells member data to companies such as Pfizer (a turn-off to some users), Within3 charges only those hospitals, charities or medical schools that use its service to create a networked sub-community, called a channel. Within3 provides tools for channel partners to document their work as well as conduct polls and surveys or share documents. To attract a wider network of PhDs as well as doctors, Within3 formed a partnership with PrometeoNetwork, a free and non-profit network of doctors and life-scientists.

PrometeoNetwork creator Giovanni Abbadessa, medical director at Ziopharm Oncology in Boston, Massachusetts, says that the network has become a way to connect clinicians with basic researchers. He plans to enable users to request job-finding services from PrometeoNetwork. Sermo's newest initiative allows users to post their comments on select journal articles from a "Discuss on Sermo" link established with the journal's publisher. (Nature Publishing Group has created links with 12 of its leading medical journals.) For example, more than 100 physicians participated in a recent discussion of research suggesting that an epilepsy drug might be useful to treat Parkinson's disease. Better communication among practitioners could help speed the translation and adoption of promising treatments.

Some sites do more than just bring people together; they let researchers share data, methodologies and protocols. MyExperiment.org, funded by the UK government, lets users share workflows: the customary protocols for standardizing data, running simulations or conducting statistical analysis on large data sets. Standardized protocols for manipulating large data sets can be tweaked for specific purposes. Users can comment on their usefulness and link to other workflows of interest. Bioinformaticians and geneticists are among those who stand to benefit most. For example, sharing a workflow for identifying biological pathways implicated in *Trypanosomiasis* resistance in cattle allowed another investigator to find pathways involved in sex dependence in the mouse model, says myExperiment project leader David De Roure, a computer scientist at the University of Southampton, UK. Done independently, this type of study could take two years. Such streamlining allows scientists to focus on discovery rather than drudgery, he says.

Tag along with this

Better yet, tagging — assigning a keyword or rating to a bookmarked online workflow or data set — allows myExperiment to connect users with similar resources that may be of interest. NanoHub, part of the NSF-funded Network for Computational Nanotechnology, lets users rate the courses and simulation tools it hosts. "In MySpace, tagging often introduces you to a new music band. On NanoHub, tagging uses the collective wisdom of the community to introduce you to appropriate simulation software," says Noshir Contractor, director of the Science of Networks in Communities lab at Northwestern University, Evanston, Illinois. He says scientists can expect more such sites streamlining their ability to find the right tools and algorithms.

Networking may get more efficient, says Contractor. Its unrealized potential is the ability to take data from networks that currently reside separately, and mash, or merge, them. He says users will soon be able to collectively mine the data of projects funded by several US agencies to see who is collaborating on what topics.

Unfortunately, some 90% of social networking sites won't succeed, says Contractor. "For every MySpace and Facebook, hundreds of others have failed," he says. Science sites that constantly update their offerings with career-enhancing capabilities have the best chance.

And of course, networking sites have their limits. Although they can facilitate connections, blogs aren't likely to become a wholesale substitute for a few beers after work any time soon. ■

Virginia Gewin is a freelancer science writer in Portland, Oregon.

D. PATITUCCI

Job-finders: Giovanni Abbadessa (left) and Massimo Pinto.

MOVERS

Josephine Briggs, director, US National Center for Complementary and Alternative Medicine, Bethesda, Maryland

2006–08: Senior scientific officer, Howard Hughes Medical Institute, Chevy Chase, Maryland
1997–2006: Director, Division of Kidney, Urologic and Hematologic Diseases, NIDDK, Bethesda, Maryland
1993–97: Professor, Departments of Internal Medicine and Physiology, University of Michigan

Since its creation in 1998, the US National Center for Complementary and Alternative Medicine (NCCAM) has fallen on tough times. It has struggled for ever-tightening funding from the National Institutes of Health (NIH), been criticized for a lack of scientific rigour, and suffered the untimely death of its first director, Stephen Straus. Staff hope that a new director will help revive its fortunes.

"Josephine Briggs will gain the staff's confidence and return stability as well as the excitement and enthusiasm needed for the centre to grow," says Ruth Kirschstein, former deputy director of the NIH and acting director of the NCCAM.

Briggs may seem an odd choice to lead the NCCAM, which oversees the development of alternative treatments. She has worked in traditional medicine since receiving her medical degree from Harvard in 1970. In fact, she specialized in renal physiology in order to work in a straightforward, quantitative field. But later career developments would pique her interest in less conventional research fare.

As a chief resident at Mount Sinai School of Medicine in New York, Briggs realized that she wanted more research training to pursue an academic career. She did a postdoc at Yale School of Medicine, working with the early leaders in evidence-based medicine, and spent six years as a research scientist at the University of Munich in Germany. Later, in the University of Michigan's nephrology division, she developed her clinical skills. She and husband Jurgen Schnermann studied the renal hormone system that helps regulate blood pressure.

She was soon made director of the kidney, urological and haematological diseases division of the National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK). While overseeing clinical trials, she noticed large placebo effects and wanted to explore them further. She had started organizing a conference on placebo effects when she discovered that NCCAM director Straus was doing the same; together, they published the conference proceedings and Briggs began to explore mind–body medicine.

She now wants to develop novel clinical-trial designs — able to detect even subtle effects — to test alternative treatments. "Many people are using these therapies, often in large amounts, and the NIH needs to do a good job of researching them," Briggs says. Kirschstein expects Briggs's background will help her better integrate complementary therapies into conventional medicine. ■
Virginia Gewin

NETWORKS & SUPPORT

Take a turn as a rotator

Interested in finding out more about the US government's science-grant review process without giving up your full-time university position? US scientists and researchers in academia and at non-profit organizations might consider taking a year or two off to work as a programme director at the US National Science Foundation (NSF). 'Temporary rotators' learn the federal grant-award process from the inside and review cutting-edge research ideas months, if not years, ahead of everyone else.

The NSF hires 100–150 rotators every year for stints lasting between one and three years, with 30–50 science and engineering positions open at any given time. These temporary programme directors (also called programme officers) are the NSF's main points of contact with the research community. They keep in touch with leading scientists, monitor emerging trends, and make funding recommendations on peer-reviewed grant proposals (see *Nature* **449,** 942–943; 2007).

"Rotators are generally experienced research scientists and engineers," says Joseph Burt, the NSF's director of human-resources management. "This means they have been published and are knowledgeable about developments in their fields."

Younger scientists and minorities are also encouraged to apply. A doctorate and at least six years' academic or non-profit research experience is required. The NSF reimburses the home institutions for the rotators' salaries, which are annualized to the calendar year.

Those interested should talk to current rotators in the field of interest to get an idea of potential staffing needs. "They welcome that contact because they are always looking to develop candidate pools, and one of the best ways is through informal communication," Burt says.

Experience as a peer reviewer often helps. Charles Conover, a physics professor at Colby College in Waterville, Maine, credits that experience for helping him win a rotator post in the NSF's physics division. "I had been involved in the programme both as a peer reviewer and as a grantee, so the people knew who I was and had a pretty good sense that I could do the writing and analysis that the job entails," Conover says. Positions are often posted in society newsletters, and society officials are good points of contact.

"We can bring folk in without a formal competition if we find the perfect fit," Burt says. ■
Ted Agres

POSTDOC JOURNAL

Our strange fellowship

I like humans' highly evolved ability to imagine. But I wish our mental hyperactivity wasn't so often a burden. Because we can imagine the worst happening — and we always do — we have vaccinations and banks and security checks and extensive visa-application processes. Everything requires long-winded translations of meaning and intent.

I've encountered plenty of this in setting up my baboon research in Ethiopia. Being an African working for an American researcher, in Africa, is not as simple as it sounds. My communication and cajoling skills have been tested to the limit while trying to arrange border crossings and behavioural research on our fellow primates. I wonder how we use so many words yet communicate so ineffectively. Why did humans not just stick with a grunt and a smile? In all my mad dashing about I felt the greatest calm at the smiles of greeting from my new employers this morning. Then we started to discuss more about long-term planning, words started flowing and things got complex once again.

For a few more days I will be surrounded by people and our world of words and paperwork and convoluted communication. I think my body has become addicted to the adrenaline necessary to survive — I sometimes like the racing heart. But I can't wait to be surrounded by primates who will simply ignore me while I search for clues about the origins of our strange habits. ■

Aliza le Roux is a postdoctoral fellow in animal behaviour at the University of Michigan.

Friedrich Miescher Institute

International PhD Programme 2008

FMI

Applications are invited for internally funded PhD student fellowships at the FMI in Basel, Switzerland. The FMI is part of the Novartis Research Foundation. Our research focuses on epigenetics, growth control and neurobiology. We employ state-of-the-art technologies to explore basic molecular mechanisms of cells and organisms in health and disease.

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Areas of focus:
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For application forms and further information, contact: phdprogramme@fmi.ch.
Application deadline: 7 April 2008

Friedrich Miescher Institute
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Maulbeerstrasse 66, 4058 Basel,
Switzerland

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UCL The Wolfson Institute for Biomedical Research

PhD STUDENTSHIPS

We are seeking applications from highly motivated candidates for a number of 3 and 4 year BBSRC PhD studentships awarded to The Wolfson Institute for Biomedical Research for October 2008.

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- Neurogenesis in the developing forebrain (Dr N Kessaris)
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- Specification of receptor tyrosine kinase (RTK) signaling. – Uncover the molecular mechanisms by which the tumour-suppressor Mig6 regulates EGF-receptor signalling (Dr I Ferby)

For information on *eligibility criteria and other project examples please visit our website http://www.ucl.ac.uk/wibr

Applications including a CV, names and addresses of two referees and a covering letter should be sent to Mrs Mandy Verdon, The Wolfson Institute for Biomedical Research, The Cruciform Building, Gower Street, London WC1E 6BT
or Email: mandy.verdon@ucl.ac.uk

The Closing date for applications is 15th March 2008

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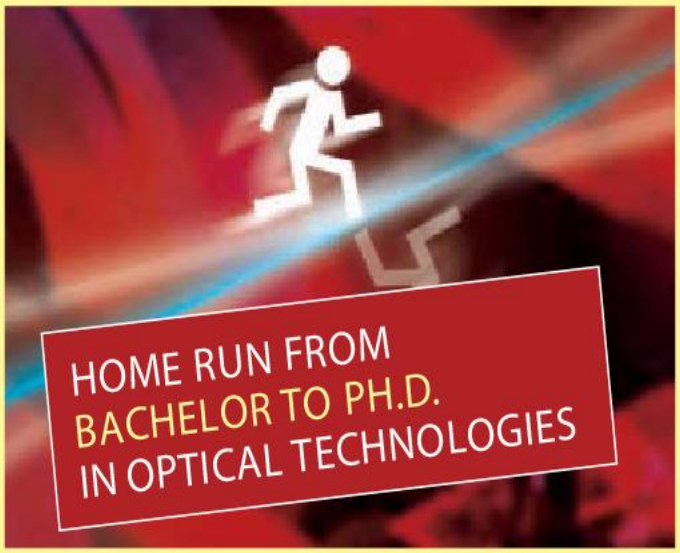

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W124991R

CBG Max Planck Institute of Molecular Cell Biology and Genetics

TECHNISCHE UNIVERSITÄT DRESDEN

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The program places great emphasis on expert thesis supervision and graduate training in an interdisciplinary context. With Dresden's strongly interconnected and world-renowned research scene and state-of-the-art facilities, the Max Planck Research School is a highly attractive and inspiring environment for young researchers.

For further details on the program, the participating research groups and the online application procedure, please visit our web page **http://www.imprs-mcbb.de**

Closing date: 1 June 2008

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invites applications for

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at the

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and its non-university partner institutes

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The graduate school places great emphasis on expert thesis supervision and graduate training in an interdisciplinary context. With Dresden's strongly interconnected and world-renowned research scene and state-of-the-art facilities, the graduate school is a highly attractive and inspiring environment for young researchers.

For further details on the program, the participating research groups and the online application procedure, please visit our web page **http://www.digs-bb.de**

Closing date: 1 June 2008

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GRK 1482

TECHNISCHE UNIVERSITÄT MÜNCHEN

The PhD Research Training Group *(DFG Graduiertenkolleg 1482)*
"Interface functions of the intestine between luminal factors and host signals"

established at the Technische Universität München (TUM) offers a distinguished PhD program, starting from May 1, 2008, to

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within a coherent research program spanning the fields of
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The PhD Research Training Group addresses specific intestinal functions in health and disease states such as transport of nutrients and non-nutrient components, gut microbial functions with challenges by pathogens and probiotics and the responses of the enteric nervous system, the intestinal and systemic immune systems and the hormonal control circuits that determine overall body homeostasis. Special emphasis is put on the role of environmental factors causing a disbalance of intestinal functions that lead and contribute to the development of chronic inflammatory bowel diseases. The program allows *state of the art research* in a cross-disciplinary fashion.

Grants will amount to €1.365 net per month (+ monthly allowance and family allowance if applicable).

TUM is an equal opportunity employer and strives to increase the percentage of female employees.

Candidates should send their applications (C.V., copies of all relevant examination certificates and academic testimonials) until **March 15, 2008** preferably by E-mail directly to the program coordinator:

Prof. Dr. Hannelore Daniel
Technische Universität München, Lehrstuhl für Ernährungsphysiologie
Am Forum 5, D-85350 Freising-Weihenstephan, grk1482@wzw.tum.de

Candidates preselected will then obtain access to the individual research projects covered in the program. They are then requested to provide a one page description for their two favourite projects in explaining why those suit them best.

Further information is available at: http://www.nutrition.tum.de/grk

W125094R

BIOZENTRUM Universität Basel

UNI BASEL

OPPORTUNITIES FOR EXCELLENCE

International PhD program at the BIOZENTRUM of the University of Basel, Switzerland

The Biozentrum together with the Werner Siemens - Foundation (WSF) launches the International PhD program in Molecular Life Sciences and encourages excellent students to apply for one of the prestigious WSF fellowships.

The Biozentrum provides an internationally renowned research environment centered around three focal areas (Infection Biology, Growth and Development, Neurobiology) and two core programs (Structural Biology & Biophysics and Computational & Systems Biology) and is dedicated to basic molecular and biomedical research (http://www.biozentrum.unibas.ch/). We offer advanced, interdisciplinary training in the field of modern biology, a lively and interactive educational atmosphere, and competitive salaries with respect to European standards. University graduates admitted to the program receive theoretical and practical training, and conduct a three-year research project under the supervision of a Biozentrum faculty member, monitored by a Thesis Advisory Committee.

Applications to the PhD fellowship program have to be submitted online. Application forms, requirements, and additional information can be found under: **http://www.biozentrum.unibas.ch/phd/.**

Application deadline: June 30, 2008

W122632R

MRC Medical Research Council | EPSRC Engineering and Physical Sciences Research Council | bbsrc Biotechnology and Biological Sciences Research Council

CARDIFF UNIVERSITY
PRIFYSGOL CAERDYDD

Research Council funded Ph.D. Studentships in Biophysics and Neuroscience

Applications are invited for six Research Council-funded Ph.D. studentships to begin in September 2008 in the School of Optometry and Vision Sciences, Cardiff University, a leading (RAE 5*A) research-led School. Fees and stipends are provided by the BBSRC, MRC and EPSRC, and are open to individuals who have been ordinarily resident in the UK for a minimum of 3 yrs on commencement of the studentship (EU-based candidates may be eligible for fees-only awards).

Research projects will investigate 1) new high-pressure cryo-vitrification approaches of corneal tissue preservation for electron microscopy (this will involve a 1-yr placement in Tohoku University, Japan), 2) molecular signatures in cornea by FTIR, 3) understanding the structural basis of pathological corneal shape changes and transparency loss, 4) molecular organisation in fibrous proteins, 5) proteoglycan biochemistry and immunochemistry in developing cornea, and 6) the modulation of eye growth by the central nervous system.

Applicants should have a first or upper second class UK Honours degree, or equivalent, in physics, the biosciences, mathematics, optometry, neuroscience, or a related discipline, and should have a desire to work alongside academic staff who are leaders in their respective fields.

Application forms can be downloaded from our website (http://www.cardiff.ac.uk) and should be returned to Mrs Sue Hobbs, School Postgraduate Secretary, indicating code AQ1 along with the project(s) which most interest you. Further information can be obtained from Dr. Andrew Quantock (QuantockAJ@cf.ac.uk).

U125544R

Doctoral Training in Medical Devices (EngD) (4 year studentships) for Engineers and Physical Scientists

University of Strathclyde

We are currently recruiting graduates in Engineering or the Physical Sciences for **October 2008** who have obtained, or expect to obtain, a first or upper second class honours degree to join the ***Medical Devices Doctoral Training Centre*** at the University of Strathclyde which is funded by the EPSRC Life Sciences Interface Programme. The centre is designed to allow graduates to carry out research relevant to problems in healthcare that can be addressed through new medical devices or related technologies. The students of the centre have the opportunity to work with medical companies and NHS and other clinical groups in state of the art research projects. The projects carried out in the centre have a high degree of relevance to the clinicians, patients and medical companies who are the end users of such research. Graduates accepted for the centre who are UK citizens will receive a four-year studentship covering living expenses and fees. EU citizens who have been resident in the UK for 3 years or more are also eligible for the full studentship. Fees only support is available for other EU citizens.

Additional information can be found at http://www.strath.ac.uk/dtc/ and http://www.strath.ac.uk/simd. An application form can be obtained from Carol McInnes Tel: 0141 548 3781 or Email: carol.b.mcinnes@strath.ac.uk.

U125787R

University of Graz
Graz University of Technology

UNI GRAZ | TU Graz

DK Molecular Enzymology

A PhD Program supported by the Austrian Science Foundation FWF

25 positions for graduate students at two universities in Graz, Austria

The aim of the international Ph.D. program in Molecular Enzymology is to provide outstanding scientific training to exceptional young scientists in different areas of the molecular biosciences, including bioorganic chemistry and biophysics. Research topics include the discovery of enzymes, enzyme structure, enzyme mechanisms, cellular and metabolic functions of enzymes, exploitation of enzymes for biotechnological and pharmaceutical applications.

For information see http://DK.uni-graz.at or contact michaela.tippl@uni-graz.at

W125550R

CAREER OPPORTUNITIES AT QUEEN'S UNIVERSITY

McClay Trust Four Year Ph.D Studentships in Cancer Research

The Centre for Cancer Research and Cell Biology, Queen's University Belfast

The McClay Trust and the Centre for Cancer Research and Cell Biology at Queen's University Belfast are delighted to announce the formation of the Sir Allen McClay Studentships in Cancer Research, and seek applications for two studentships commencing September 2008.

Sir Allen McClay is Founder and Chairman of the Almac Group, a Northern Ireland-based group of companies undertaking innovative R&D and service provision globally in the pharmaceutical, biotechnology and diagnostic sectors (www.almacgroup.com). Sir Allen has also established the McClay Trust, a charitable body whose principal objectives are to support research and development activities within Queen's University. To date the Trust has committed approximately £20 million to the University in support of these activities.

The Centre for Cancer Research and Cell Biology (CCRCB) at Queen's University Belfast is an interdisciplinary centre of international research excellence focused on cancer and infectious disease. Underpinned by a £25M investment in personnel and infrastructure, the CCRCB is home to over 45 research groups, undertaking basic scientific, translational and clinical research. A strong research-based postgraduate education and training programme is at the core of the Centre's research activities.

Each Sir Allen McClay Studentship will pay the student's tuition fees, provide an enhanced stipend of £16,000 per annum, and provide £10,000 in research consumables per annum. The student will undertake a mentored research project towards the award of a Ph.D degree over four years. Students will select a laboratory and supervisor at the end of the first year following a series of laboratory rotations in the Cancer Biology, Infection and Immunity and Biomedicinal Chemistry/Experimental Medicine research divisions.

Applications are invited from EU residents for these Ph.D studentships. Successful applicants should hold or expect to attain a First Class or Upper Second Class Honours Degree (or equivalent) in Biochemistry, Molecular Biology or closely related subject and will be highly motivated individuals eager to undertake scientific research in a dynamic research environment. Evidence of laboratory-based biomedical research experience is desirable.

To apply, please forward a copy of your Curriculum Vitae including a synopsis of research experience together with the names and addresses of two academic referees to Nancy Bowman, School of Biomedical Sciences, Queen's University Belfast, 97 Lisburn Road, Belfast BT9 7BL or e-mail n.bowman@qub.ac.uk by **Friday 14th March, 2008 at 5.00pm.**

For more information on CCRCB Staff and Research, the Four Year Ph.D programme and the Sir Allen McClay Studentships, please consult our website (www.qub.ac.uk/ccrcb).

Queen's University Belfast

Rg Queen's University Belfast is a member of the Russell Group of universities. One of the United Kingdom's top 20 research-intensive universities.

U125773R

KING'S College LONDON

U125772RL

Centre for Bioinformatics
Computational Genomics
PhD Studentship

PhD to address the large-scale analysis of cellular networks involving mathematical and statistical modelling, database technologies and related computational biology software.
The work will follow on approaches for module detection and data mining of protein interaction networks to enhance understanding of molecular interactions and evolution.

Contact
Dr Sophia Tsoka
Tel: +44 (0)20 7848 1056
Email: bioinformatics@kcl.ac.uk
www.kcl.ac.uk/schools/pse/bioinform/

UNIVERSITY OF BATH

Department of Pharmacy & Pharmacology

PhD studentship programme

Opportunities exist for enthusiastic, high quality post-graduate students to join the RAE 5*A-rated Department of Pharmacy and Pharmacology, one of the leading departments in the UK. The department has an international reputation for research involving multi-interdisciplinary approaches to cutting edge questions in drug target identification and validation, drug discovery, drug delivery and drug action. Current research interests encompass the following areas:

- inflammation, cancer, infectious diseases, regenerative medicine
- molecular signalling and cell biology
- vascular and neuropharmacology
- chemical biology, medicinal chemistry
- drug delivery
- pharmacoepidemiology
- health psychology
- clinical pharmacy

We are inviting applications for PhD studentships (open to EU and non-EU citizens) commencing September 2008.

Further details of our scientific programmes and project leader contact details can be found via http://www.bath.ac.uk/pharmacy//research/index.shtml. Applicants should have, or expect to gain, good degrees (minimum 2:1) in any relevant discipline. Informal enquiries are encouraged and should be directed to individual project leaders (see website for contact details). Formal application information is available at http://www.bath.ac.uk/pharmacy/postgraduates/howToapplyRes.shtml.

U125559R

www.bath.ac.uk/jobs

UCL

UCL Department of Biology

Potential PhD studentships available from September 2008

Molecular and cellular basis of sexual traits in stalk-eyed flies - Dr K Fowler (k.fowler@ucl.ac.uk)

Genetics of longevity and ageing in the nematode C. elegans - Dr D Gems (david.gems@ucl.ac.uk)

Analytical tools and strategies for genome-wide association mapping of disease genes - Dr N Maniatis (n.maniatis@ucl.ac.uk)

Understanding the genomic program for early embryo development - Dr P Oliveri (p.oliveri@ucl.ac.uk)

Signalling pathways in the extension of lifespan by dietary restriction - Prof L Partridge (l.partridge@ucl.ac.uk)

Mosses and atmospheric ammonia pollution - Dr J Pearson (john.pearson@ucl.ac.uk)

Synthesis of human proteins in green algae - Dr S Purton (s.purton@ucl.ac.uk)

Sexual antagonism in fruitflies - Dr M Reuter (m.reuter@ucl.ac.uk)

Structures and mechanisms of mitochondrial electron transfer proteins with infrared vibrational spectroscopy - Prof P Rich (prr@ucl.ac.uk)

Distribution of variation in sub-Saharan Africa in genes coding for P450 drug metabolising enzymes - Prof A Ruiz Linares (a.ruizlin@ucl.ac.uk)

Genetic basis of the response of plants to their environment - Dr A Wingler (a.wingler@ucl.ac.uk)

Funding is from UK Research Councils. Contact the appropriate supervisors for information. Applications with CV and details of two referees to go to the appropriate supervisor. Applicants must hold, or expect, a First or Upper Second Class honours degree in an appropriate subject. Applicants must meet residency criteria of Research Councils to receive full studentships. Contact details and abstracts available at: http://www.ucl.ac.uk/biology/grads/phd-positions/phd-places.html

Closing date: 19th March 2008.

U125754R

Biostatistics Bioinformatics Unit

CARDIFF UNIVERSITY
PRIFYSGOL CAERDYDD

MSc in Bioinformatics or Genetic Epidemiology and Bioinformatics

Masters Course Bursary Fund

Led by The Biostatistics and Bioinformatics Unit (BBU; http://bbu.cardiff.ac.uk), in close collaboration with five internationally recognised cancer research groups and Cancer Research UK, this scheme provides generous bursaries for cancer researchers (or potential future cancer researchers) to study on our established MSc courses.

Three bursaries are available to commence this Autumn and include full payment of Home/EU student fees plus a stipend towards living expenses (£5500). Please note that individuals with other interests are encouraged to apply for these courses but are not eligible for these bursaries.

For further details on these bursaries and how to apply please refer to our website or contact Michelle McDonald (mcdonaldmd@cardiff.ac.uk).

Deadline for application: 1st June.

CANCER RESEARCH UK

U123036R

SFB 575

Scholarships for Doctoral Dissertation (Ph.D./M.D.) in the Integrated Research Training Group

HEINRICH HEINE UNIVERSITÄT DÜSSELDORF

The Collaborative Research Center SFB575 "Experimental Hepatology" (head: Prof. Dr. D. Häussinger) at the University of Duesseldorf in Germany, announces open "Scholarships for Doctoral Dissertation" in the field of Medicine (Internal Medicine, Neurology), Biochemistry, Neuroscience, Biology, Chemistry and Physical Chemistry.

For detailed information please visit our website www.uni-duesseldorf.de/sfb575
Please download our application form in addition to a detailed letter of motivation and mail it to: sfb-575.igrk@med.uni-duesseldorf.de

W125440R

The Bloomsbury Colleges PhD Studentships

The Bloomsbury Colleges are a consortium of six University of London colleges comprising Birkbeck (BBK), Institute of Education (IoE), London School of Hygiene & Tropical Medicine (LSHTM), Royal Veterinary College (RVC), School of Oriental and African Studies (SOAS) and The School of Pharmacy (SoP).

The Consortium is offering twelve PhD Studentships, each of three years duration, to start October 2008. The Studentships will cover course fees (at the usual level for UK and EU Studentships) and a student stipend. They will encompass a wide range of topics spanning the biomedical and social sciences, reflecting the diversity of disciplines represented in the consortium.

The Studentships will be supervised by two of the partner colleges with the lead college indicated after each Studentship.

The Studentships available are as follows:

- Legalisation and judicialisation in the provision of health and care services. - BBK
- Using technology to detect and improve social deficits in children with Autism. - BBK
- Medicines for Children: priorities for research and outcome assessment. - IoE
- School experiences and educational attainment for children in rural compared with urban areas. - IoE
- Active ageing, social engagement and health of older people in Europe: population and policy influences. - LSHTM
- *Escherichia coli* K1 interactions with human brain microvascular endothelial cells, a primary step in the development of Neonatal Meningitis. - LSHTM
- Investigations into human urothelial cell signalling pathways and antibiotic response in Overactive Bladder (OAB). - RVC
- New antibacterials from plants and their modes of action. - RVC
- The investment behaviour of Chinese listed firms: how does it differ from UK firms? - SOAS
- Too soon for an obituary: clinical legal education in the English law school, and the prospects for experiential learning in international human rights. - SOAS
- Comparative oncology investigation of membrane active polymers for the treatment of cancer. - SoP
- Structural studies of a kinesin adaptor protein: an important mediator of trafficking processes in neurons. - SoP

Further details are available from www.bloomsbury.ac.uk/studentships. Closing Date: 14 March 2008.

U125821R

Berlin, Charité

International Graduate Program Medical Neurosciences

NEUROCURE
Towards a better outcome of neurological disorders

PhD positions in neuroscience

We invite applications for PhD positions in neuroscience.

The 25 internationally recognized principal investigators of NeuroCure cover a large range of topics from basic molecular and cellular to clinical neuroscience with a strong emphasis on translational research.

Established research topics are neural plasticity, mechanisms of damage, endogenous brain protection, regeneration, developmental disturbances and crosstalk between nervous and immune system. The clinical focus is on cerebrovascular diseases, neuroinflammation, disorders of network formation, stroke, multiple sclerosis, focal epilepsies and disturbances in mitochondrial dysfunctions.

We look for outstanding, highly motivated candidates of any nationality, who hold or expect a Masters degree or equivalent in neuroscience, biology, chemistry, physics or a related field. The working language of the program is English. To learn more about NeuroCure and the application process please visit: **www.neurocure.de**

NeuroCure collaborates with the International Graduate Program Medical Neurosciences of Charité – www.medical-neurosciences.de

Application closing date: April 15, 2008 | Starting date: summer 2008

W125069R

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Faculty of Applied Sciences, TU Delft
Netherlands

Departments

- Biotechnology
- Chemical Engineering
- Imaging Science and Technology
- Multi-Scale Physics
- Nanoscience
- Radiation, Radionuclides and Reactors

Various paid PhD positions are available at our faculty

For current PhD positions look at www.tnw.tudelft.nl/phd

TU Delft
Delft University of Technology

W125080R

Werner Reichardt

Centrum für Integrative Neurowissenschaften

EBERHARD KARLS UNIVERSITÄT TÜBINGEN

The Werner Reichardt Centre for Integrative Neuroscience (CIN) is a newly established interdisciplinary institution at the Eberhard Karls University Tübingen funded by the German Excellence Initiative programme. It is supported by 6 faculties (Biology, Medicine, Mathematics and Physics, Computer and Cognitive Sciences, History and Philosophy, Modern Languages), the Max Planck Institute for Biological Cybernetics, the Hertie Institute for Clinical Brain Research, the Fraunhofer Institute for Manufacturing Engineering and Automation, and numerous partners in the industry. The CIN strives to deepen our understanding of how the brain generates function and how brain diseases impair functions. It will make use of newly acquired insights to help people with brain disorders and to launch new mind- and brain-inspired applications in many areas of engineering and computer science.
Its scientific programme is guided by the conviction that progress in the understanding of brain function can only be achieved with an integrative approach spanning multiple levels of organization and pooling the knowledge of researchers from many different fields.
In order to strengthen specific research areas and approaches, the CIN is now offering a number of junior group leader (JRG) positions with tenure track options for young scientists with a promising track record. The positions at the CIN will be complemented by two additional JRG positions available at the **Hertie Institute for Clinical Brain Research (HIH)**.

Junior Research Groups at the Werner Reichardt Centre for Integrative Neuroscience Tübingen

JRG on "Auditory Brainstem Implants"
This JRG will work on the development and evaluation of an auditory brain stem implant, restoring residual hearing in patients who are deaf as a result of destruction of the inner ear and / or the auditory nerves.

JRG on "Neurophilosophy"
This JRG is expected to apply findings from neuroscience to traditional philosophical questions. Neurophilosophical answers to these questions should be informed by empirical findings about the neural realisations of psychological states.

JRG on "Synaptic Plasticity"
This JRG will address the molecular, cellular or functional basis of synaptic plasticity and its role in memory formation, learning and the recovery from stroke or other forms of brain disease. The area of research may also include the causative role of altered synaptic plasticity in brain disease as well as functional and dysfunctional adaptations to neurodegenerative disease.

JRG on "Genetic Tools"
This JRG will explore the potential of genetic manipulations in unveiling the principles behind information processing on the cellular and network level in-vivo. Preferably such technology should be directly applicable to the adult brain e.g. using viral vectors and RNA interference technologies.

JRG on "Brain Oscillations and Animal MEG"
This JRG will investigate the role of oscillatory brain activity in cortical information processing using non-human primates as models of the human brain. The successful applicant should have a proven track record in the study of oscillatory brain activity and the synchronization of neuronal activity in animals. In addition, experience in MEG-based studies of oscillatory brain activity in humans is highly desirable. The applicant will be expected to set up and operate an animal MEG system in order to combine invasive and MEG approaches.

JRG on "Central Visual Prosthetics"
The JRG will embark on studies of cortical visual processing in an attempt to lay the groundwork of basic research for the development of a visual prosthesis interfacing the visual cortex and restoring some aspects of natural vision in blind patients. Potential topics to be addressed by this group could include the role of fixational eye movements for the deconvolution of image information or the optimization of high resolution microstimulation hardware and software.

JRG on "Infant Cognition"
This JRG will study the development of human cognition. Psychological investigations are to be combined with non-invasive measurements of brain activity.

JRG on "Optogenetics"
This JRG is expected to explore the potential of converting inner retinal neurons to photosensitive cells e.g. by incorporating light-gated channels into neurons with the aim of testing whether the resulting light-sensitisation gives rise to ecologically relevant visual percepts.

JRG on "Medical Robotics"
This JRG will develop robotic systems to support the analysis, treatment and rehabilitation of patients with motor deficits and neurological disorders at large and it may focus on the construction of novel robotic devices, suitable for application to and measuring of the forces and dynamic parameters of motor behaviour.

JRG "Free floater" groups for junior female neuroscientists
The CIN is firmly committed to gender equality, and encourages junior female neuroscientists to submit proposals for additional junior research groups on their topic of choice.

JRG on "In vivo optical imaging of the brain" (HIH)
The HIH is seeking an individual to apply and refine the usage of cutting edge optical techniques in studies of the intact brain on the level of cells and networks of cells. Applicants should have a solid background in nonlinear optical microscopy such as two photon microscopy and a proven interest in applying these techniques to problems in basic and/ or clinical neurosciences. The successful candidate is expected to show proven potential to develop an independent research program that may address fundamental issues in any aspect of the neurosciences. However, independent of the research focus of choice, she/ he should be willing to disseminate optical techniques to interested groups at the HIH and to develop strong interdisciplinary interactions.

JRG on "The Molecular Basis of Memory"(HIH)
We expect this JRG to study the molecular mechanisms underlying learning and memory in suitable model organisms.

Framework: Each position is scheduled to run for 5 years with evaluations by external experts at regular intervals. JRG receiving positive evaluations after 3 years may obtain a **tenure track** option which may ultimately lead to a **professorship** at the CIN/HIH. Start-up funds as well as substantial funding for personnel and running costs will be available for all new groups and depend on the qualification and prior experience of the applicant. Appointees will be full members and active participants in the CIN, which will also provide laboratory space. JRG leaders will be provided opportunities to contribute to research-oriented training within the framework of the CIN Graduate Training Centre and the faculties involved in the CIN will provide opportunities for the German habilitation according to established rules, if desired. According to German law, severely disabled persons with equal occupational aptitude will be given preferential consideration. The University of Tübingen strives to promote equal opportunities in science and is committed to increasing the percentage of female scientists in teaching and research. Qualified female candidates are thus strongly encouraged to apply.
Applicants should clearly identify the JRG topic of their choice, submit a curriculum vitae, pdf files of up to 5 key publications, statements of research achievements and future directions (not to exceed 3 pages) as well as the names and addresses of at least three referees. All documents should be submitted electronically within four weeks after publication of this advertisement to the Acting Director of the Werner Reichardt Centre for Integrative Neuroscience Tübingen, Prof. Dr. Peter Thier, at **cin@uni-tuebingen.de**.
For further information on the CIN see: http://www.uni-tuebingen.de/CIN.

W125444R

Newcastle University

Senior Research Associate & Research Associates (3 Posts) £25,134 - £35,837 p.a.

Applications are sought for 3 postdoctoral bio-scientist positions to undertake drug discovery research in a highly successful multidisciplinary research group with a proven track-record of drug development, particularly the identification of inhibitors of DNA repair pathways and other targeted anticancer drugs. Based in the Northern Institute for Cancer Research at Newcastle University, you will join a vibrant team of over 20 researchers developing new cancer drugs designed to exploit underlying molecular defects in a range of human cancers. The research will be conducted in the new £10M Paul O'Gorman Building and is supported by a major programme grant from Cancer Research UK, as well as by pharmaceutical and biotech companies. There is access to a wide-range of contemporary laboratory equipment, including non-invasive imaging facilities.

Post 1: Senior Research Associate (Molecular Pharmacologist: Supported by Cancer Research UK)

This post requires the use of contemporary molecular biology techniques to identify and exploit novel targets for anticancer drug discovery. Investigations in cells over-expressing or rendered defective in the target (through genetic manipulation or knock-down) will be conducted. Methods to measure the cellular activity of the target will be developed to allow cell-based inhibition assays to be conducted. These assays will be developed into pharmacodynamic biomarkers for agents progressing to clinical trials. Previous experience of a wide-range of contemporary cell biology and molecular biology techniques is essential, preferably in a drug discovery setting. As well as conducting research on specific projects, the researcher will train, supervise, advise and aid other researchers within the team.

Job reference: A030R.

Post 2: Research Associate (Pharmacologist: Supported by Cancer Research UK)

The post requires the use of pharmacological techniques to evaluate novel anticancer drugs at the lead optimization stage of drug discovery. Pharmacokinetic, pharmacodynamic and antitumour studies will be conducted using a wide-range of models, applied to diverse and innovative targets. Previous experience of pharmacological studies, preferably as applied to cancer drug discovery, is essential.

Job reference: A031R.

Informal approaches for the above positions can be made to Professor Nicola Curtin, n.j.curtin@ncl.ac.uk

Post 3:Research Associate (Pharmacologist: Supported by the European Commission)

The post also requires the use of pharmacological techniques to evaluate drugs designed to disrupt protein-protein interactions for the treatment of brain tumours. The research will be undertaken as part of a consortium of 7 academic and commercial European groups, in which the Northern Institute for Cancer Research is responsible for the synthesis and biological evaluation of potential drugs. Previous experience of pharmacological studies, preferably as applied to cancer drug discovery, is essential.

Job reference: A028R.

Informal approaches for the above post can be made to Professor Herbie Newell, herbie.newell@ncl.ac.uk

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Closing date: 19/03/08.

To apply for any of the above positions you should submit a covering letter, an up-to-date CV giving full details of your qualifications and experience, as well as a completed Employment Record Form and quoting the appropriate Job Reference Number, to Mrs Sandra Cartwright, Institute Secretry, Northern Institute for Cancer Research, Newcastle University, Paul O'Gorman Building, Medical School, Framlington Place, Newcastle upon Tyne, NE2 4HH.

U125823R

Committed to Equal Opportunities www.ncl.ac.uk/vacancies

3 Posts in NIHR Biomedical Research Centre

A new specialist NIHR Biomedical Research Centre for Mental Health has recently been established at the Institute of Psychiatry (King's College London) and South London and Maudsley NHS Foundation Trust (SLaM). The Centre has been funded competitively through the National Institute for Health Research to undertake 'translational research' that will help patients benefit more quickly from new scientific breakthroughs.

Bioinformatician

Ref: 08/R18

You will be responsible for database creation as well as analyses for genetics, genomics, transcriptomics and proteomic data alongside data from neuroimaging, laboratory and clinical areas. The post is within the Biomedical Technologies theme of the NIHR Biomedical Research Centre for Mental Health. You will be expected to take a leading role in the analysis of this data as well the development of new techniques and will therefore need to be familiar with relevant database languages and statistical programmes; including MYSQL, PERL and R. Expertise in expression and genetic analysis would be desirable as well as enthusiasm for bioinformatic exploratory analyses.

Starting salary in the range £23,800 - £29,716 per annum (inclusive of £2,323 pa London Allowance), depending on qualifications and experience.

Mass Spectrometrist

Ref: 08/R19

We are looking for an enthusiastic person to provide assistance with proteomics research, in particular with mass spectrometry, to the NIHR Biomedical Research centre for Mental Health. Such assistance will include providing advice, technical assistance, technology development and conducting own-research relevant to the centre. You will be a full member of the proteomics facility at the Institute of Psychiatry which collaborates with and provides core proteomics expertise to groups within KCL, including other research groups at the IoP especially the MRC Centre for Neurodegeneration Research

Starting salary in the range £23,800 - £29,716 per annum (inclusive of £2,323 pa London Allowance), depending on qualifications and experience.

Laboratory Assistant

Ref: 08/T02

You will be responsible for samples management and central lab duties within the NIHR Biomedical Research Centre for Mental Health. You will be expected to take a co-ordinating role in curating and maintaining sample collections, DNA handling and mRNA extraction as well as genotyping of SNPs and VNTRs and possible QPCR. Database for sample tracking and data entry and storage will be a feature of the post. Experience in expression and genetic analysis would be desirable and well as enthusiasm for laboratory work.

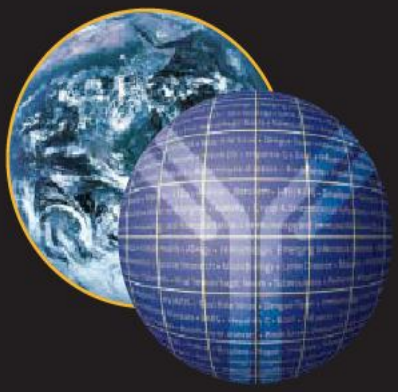
Starting salary in the range £22,915 - £28,278 per annum (inclusive of £2,456 pa London Allowance), depending on qualifications and experience.

To obtain further particulars, an application form and further information about the Institute, please see our website at http://www.iop.kcl.ac.uk/vacancies email vacancies@iop.kcl.ac.uk Completed applications should be emailed or posted to the addresses given in the Further Particulars. Please quote the appropriate reference numbers in all correspondence.
Closing date : 14 March 2008.

Equality of opportunity is College policy

KING'S College LONDON

U125767R



NIAID

NATIONAL INSTITUTE OF ALLERGY AND INFECTIOUS DISEASES

Help Us Help Millions

Clinical Tenure-Track Position

The National Institute of Allergy & Infectious Diseases (NIAID), Division of Intramural Research (DIR), is seeking an outstanding tenure-track investigator to develop a clinical research program to better understand, treat, and ultimately prevent infectious, immunologic, and/or allergic diseases. The scope of the NIAID research portfolio has expanded considerably in recent years in response to new challenges such as bioterrorism; emerging and reemerging infectious diseases, including acquired immunodeficiency syndrome (AIDS), influenza, severe acute respiratory syndrome (SARS), West Nile virus, malaria, and tuberculosis; immunologic diseases and the increase in asthma prevalence among children in this country.

The successful candidate will implement and direct an independent clinical research program with research emphasis on clinical research but may include translational or basic research. The incumbent will have the opportunity to choose the Laboratory in which he/she would like to be affiliated. It is expected that clinical protocols developed will complement the research goals of the Laboratory selected. In addition, the candidate will be paired with a Senior Investigator who will serve as a clinical mentor.

An outstanding postdoctoral record of research accomplishment and M.D., M.D. /Ph.D. or equivalent degree is required for this position; board eligibility/board certification is also required. The incumbent will be expected to be qualified for credentialing by the NIH Clinical Center.

Candidates will be assigned independent resources to include clinical and/or laboratory support personnel, equipment, space, and an allocated annual budget for services, supplies, and salaries to ensure success. This is a tenure-track appointment under Title 42. Salary is dependent on experience and qualifications.

Interested candidates may contact Dr. Karyl Barron, Deputy Director, DIR, NIAID at 301/402-2208 or email (kbarron@nih.gov) for additional information about the position.

To apply for the position, send your curriculum vitae, bibliography, and an outline of your proposed research program (no more than two pages), by **March 14, 2008** via email to Ms. Wanda Jackson at jacksonwa@niaid.nih.gov. In addition, three letters of recommendation must be sent to Chair, NIAID DIR Clinical Tenure Track Search Committee, c/o Ms. Wanda Jackson at jacksonwa@niaid.nih.gov or 10 Center Drive MSC 1356, Building 10, Rm. 4A-26, Bethesda, Maryland 20892-1356. E-mail is preferred. Please note search #018 when sending materials.

Further information regarding the DIR laboratories is available at: http://www3.niaid.nih.gov/about/organization/dir/default.htm and information on working at NIAID is available on our website at: http://healthresearch.niaid.nih.gov/tdir

Department of Health and Human Services
National Institutes of Health
National Institute of Allergy and Infectious Diseases

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OPPORTUNITIES @ NIH THE NATIONAL INSTITUTES OF HEALTH

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University of Oxford

Nuffield Department of Clinical Medicine

Non-Clinical NDM Scientific Leadership Fellows

In the areas of Respiratory Medicine, Human Genetics, Neuroscience, Genomic Epidemiology, Cryo-Electron Microscopy, Angiogenesis, Cancer Research, Pathogen Research and Diabetes, Endocrinology and Metabolism

The Nuffield Department of Medicine is a large multi-disciplinary department hosting one of the largest groupings of biomedical sciences in the University sector. It was awarded the top five-star ranking in each of the last two Research Assessment Exercises and has external research grant support in excess of £64m per annum. The Department has developed strong programmes in molecular and cell biology, structural biology, genetics and genomic medicine, proteomics, large-scale epidemiology and clinical trials and experimental medicine (www.ndm.ox.ac.uk). The Nuffield Department of Medicine invites applications for the current round of Scientific Leadership Fellows in the following fields and intends to appoint to seven posts.

Wellcome Trust Centre for Human Genetics www.well.ox.ac.uk

In any of the following fields: Neuroscience, Cell Biology, Chromatin Biology/Epigenetics, Functional Genomics or Statistical Genetics/Genetic Epidemiology.

Enquiries: Professor Jonathan Flint by e-mail: jf@well.ox.ac.uk

MRC Centre for Genomics and Global Health www.cggh.ox.ac.uk

In the field of genomic epidemiology, encompassing statistical genetics, bioinformatics and population genetics applied to malaria and other major diseases of the developing world.

Enquiries: Professor Dominic Kwiatkowski by e-mail: dominic@well.ox.ac.uk

Structural Biology Division www.strubi.ox.ac.uk

In the following field: Cryo-Electron Microscopy/Tomography.

Enquiries: Professor Dave Stuart by e-mail: dave@strubi.ox.ac.uk

Centre for Cellular & Molecular Physiology www.ccmp.ox.ac.uk

In the following field: Angiogenesis.

Enquiries: Professor Peter Ratcliffe by e-mail: pjr@well.ox.ac.uk

Ludwig Institute for Cancer Research http://www.licr.org

In any of the following fields: Cancer Stem Cells, Metastasis, Tumour Suppression, Senescence or Cancer Metabolism.

Enquiries: Professor Xin Lu by e-mail: x.lu@ludwig.ox.ac.uk

Respiratory Medicine

In the field of Respiratory Epidemiology/Statistics, Cell Biology or Lung Infection.

Enquiries: Dr R Davies by e-mail: robert.davies@ndm.ox.ac.uk

Oxford Centre for Diabetes and Endocrinology & Metabolism www.ocdem.ox.ac.uk

In any of the following fields: Islet Cell Biology, Nuclear Receptor Biology or Translational Biology.

Enquiries: Professor Mark McCarthy by e-mail: mark.mccarthy@drl.ox.ac.uk

The purpose of this scheme is to provide an efficient entry point to this environment for non-clinical basic scientists wishing to establish a career within the UK as a principal investigator. It is intended that the scheme will suit those with between three and eight years postdoctoral experience who would, once established, be in a good position to enter one of the UK's established senior fellowship schemes funded by external bodies, such as the Wellcome Trust, the MRC, the Royal Society, the BBSRC, CRUK or the BHF.

The NDM Scientific Leadership Scheme will support: (1) Personal salary for up to three years, (2) Funding for a 'start-up' postgraduate or postdoctoral research assistant working with the fellow for two years and (3) Start-up consumables & small equipment expenses.

To apply please send a detailed CV and a short statement of current research interests, the contact details of two professional referees (one of whom must be the current employer) to the Personnel Administrator, Wellcome Trust Centre for Human Genetics, Roosevelt Drive, Oxford OX3 7BN (e-mail: personnel@well.ox.ac.uk or fax. (01865) 287516. Please quote reference H5-08-021-SL. The closing date for applications is 20 March 2008.

As an Equal Opportunity employer, we positively encourage applications from people of all backgrounds

U125804RM

www.ox.ac.uk/jobs

MANCHESTER 1824

The University of Manchester

Faculty of Life Sciences

5 Year Manchester Life Sciences Fellowships (3 posts)

£26,666- £41,545 p.a. **Ref: LS/025/08**

We are pleased to offer three highly prestigious 5 year Fellowships suitable for outstanding researchers in life sciences. Applications are invited from progressive bioscientists with strong CVs in cardiovascular sciences, tissue engineering, neuroscience or physiological systems. Proposals from researchers specializing in translational biology or medicine in any of the aforementioned disciplines are especially encouraged.

You will be expected to form links with existing principal investigators within the Faculty of Life Sciences (RAE 5*), and colleagues in the Faculty of Medical and Human Sciences. It is envisaged that fellows will be independent by the end of the 5 year award. The collegiate nature of the University encourages collaboration and exchange, and your research aspirations will be furthered by existing strengths in stem cell research, membrane proteins, tissue engineering and extracellular matrix research.

Informal enquiries can be made to Dr Craig P Smith on +44 (0) 161 275 5460 or craig.smith@manchester.ac.uk

Application forms and further particulars are available from our website or by contacting +44 (0) 161 275 8836 or Lifesciences-hr@manchester.ac.uk quoting the reference number.

Closing date: 13 March 2008.

School of Clinical & Laboratory Sciences

Research Facilitator

£26,666 - £32,796 p.a. **Ref: MHS/064/08**

You will be a highly motivated, enthusiastic individual with research training to at least PhD level in biological sciences, ideally including post-doctoral research. Previous research experience/interest in reproduction (major pregnancy complications, pre-eclampsia, premature labour, foetal growth restriction, stillbirth etc) is desirable but not essential.

Duties will involve the preparation and submission of grant applications, publications and ethical applications and co-ordination of clinical studies. You will be required to aid in supervision of graduates and postgraduate students and be a contact point for all grant and ethical queries. The ability to advise and support other scientists in preparing oral and written presentations, scientific papers, thesis' and written reports is essential.

This post would be suitable for someone considering a future career in research administration or scientific/medical writing.

This post is tenable for of two years.

Informal enquiries can be made to Professor Phil Baker on +44 (0) 161 276 5460.

Application forms and further particulars are available from our website or by contacting +44 (0) 161 275 1197 or julie.heydon@manchester.ac.uk quoting the reference number.

Closing date: 7 March 2008.

The University will actively foster a culture of inclusion and diversity and will seek to achieve true equality of opportunity for all members of its community.

U125775R

www.manchester.ac.uk/jobs

Evolving the Future of Drug Discovery and Development

MedImmune, the global biologics unit of AstraZeneca plc, is committed to advancing science for better health. To help us sustain our goal of leadership in discovery technologies, we have two opportunities to join our exceptional Platform Development team in Cambridge, United Kingdom.

Principal Peptide Chemist Cambridge, United Kingdom

Acting as the scientific leader for peptide chemistry at MedImmune, you will set the chemistry strategy for relevant projects and provide significant input into their overall scientific direction. In particular, you will be responsible for the design and synthesis of peptides during therapeutic programs. You will work closely with molecular biologists, expert in Ribosome Display, to establish methods for synthesis of cyclic peptides, design peptide libraries incorporating unnatural amino acids/pharmocophores for Ribosome Display and ensure that MedImmune has access to the most advanced chemistry compatible with its display technologies. You will have a PhD in your relevant field, with post-doctoral experience including responsibility for directing own project; experience of leading a small team would be desirable. You will have experience in peptide chemistry and peptide synthesis and the capability to devise syntheses using unnatural amino acids and pharmacophores. An understanding of the principles of pharmacology and medicinal chemistry would be advantageous. In addition you will have an enthusiastic approach to science and the ability to efficiently communicate your results and ideas to colleagues from different disciplines.

Research Scientist Peptide Chemist Cambridge, United Kingdom

Reporting to the Principal Peptide Chemist, your role as Research Scientist will involve providing synthetic chemistry expertise to design and synthesize novel peptide therapeutics in conjunction with molecular evolution techniques. Educated to PhD level you will have a successful record of independently planned research work and experience in peptide chemistry and peptide synthesis. Knowledge of protein and peptide structure analysis along with an understanding of translation and/or molecular biology is desirable.

In all cases we are looking for people who will be committed to helping us achieve our ambitious goals – proactive team players with a meticulous logical and analytical approach to problem solving, good interpersonal skills and a passion for personal development. Key skills in working collaboratively are of particular interest.

To find out further details, and to apply, please go to the MedImmune Cambridge (formerly Cambridge Antibody Technology) website: www.cambridgeantibody.com/home/careers_@_cat

MedImmune is an Equal Opportunities Employer, we seek to empower each individual, and respect the diverse cultures, perspectives, skills and experiences within our workplace.

MedImmune

U125757R

wellcome trust sanger institute

The Wellcome Trust Sanger Institute
Human Genetics
Postdoctoral Fellows

Postdoctoral fellows in human genetics to research projects on genetics of human diseases, see www.leenapeltonen.eu or www.ncoedg.org. Our activities are in the field of human genetics including genome-scale resequencing large scale genotyping and association studies, genome-wide copy number analyses, searches for predisposing alleles in diseases.

Contact

Email: Recruit@sanger.ac.uk
http://www.sanger.ac.uk

U125678RL

UNIVERSITÄT BASEL

The **Biozentrum** of the University of Basel, Switzerland (http://www.biozentrum.unibas.ch) invites applications for the tenured position of

A Full Professor in Structural Biology (Electron Microscopy).

The position will have tight connections with **SystemsX.ch,** the Swiss initiative for Systems Biology and provides an exceptional opportunity for internationally recognized scientists to develop cutting edge research in structural biology. The equipment available allows to perform TEM, STEM, atomic force microscopy, single particle cryo EM and electron crystallography. A new 300 kV instrument for cryo electron tomography of vitrified cells has been ordered.

The Biozentrum integrates Structural Biology & Biophysics and Computational & Systems Biology with three focused Research topics (Cell Growth & Development, Infection Biology and Neurobiology) and offers exceptional opportunities for multidisciplinary interactions.

Applications including a motivation letter (max 2 pages), curriculum vitae, a description of research plans and the names of three references should be sent by **April 1st, 2008** to Prof. Dr. Hans-Peter Hauri, Dean, Faculty of Science, University of Basel, Klingelbergstrasse 50, 4056 Basel, Switzerland. The University of Basel is an equal opportunity employer. Applications from women candidates are particularly encouraged. Applications should also be sent electronically to Marianne.Hess@unibas.ch.

UNI BASEL

W125692R

CHARITÉ - UNIVERSITÄTSMEDIZIN BERLIN

W125747R

The Institute of Neuropathology of the Charité is offering the following position:

Senior Postdoctoral Fellow (Reference Number: DM.09.08)

We offer a dynamic and growing environment specialised in neurological disease research at the interface of neuropathological diagnostic in a leading European university clinic.

⇨ ***Tasks:***
Scientific research in a dynamic and exciting neuroscientific environment at the interface between fundamental and clinically-applied research related to immunological aspects of neurological degenerative diseases and especially to Alzheimer's disease.

⇨ ***Prerequisites and qualifications:***
- Postgraduate in biology, biochemistry, medicine, or related disciplines
- PhD or doctorate
- highly interested in neurosciences
- Experience with molecular, biochemical and immunological techniques, handling laboratory animals/mice and in generating genetically modified mice

Please send your application to **Prof. Dr. F. Heppner, Institut für Neuropathologie, Charité - Universitätsmedizin Berlin, Augustenburger Platz 1, 13353 Berlin.** For further information, contact us at: (+49) 30 450 536042.

MRC Cancer Cell Unit

Hutchison/MRC Research Centre
MRC Cancer Cell Unit

The Hutchison/MRC Research Centre (www.hutchison-mrc.cam.ac.uk) is an established centre for cancer research alongside Addenbrooke's Hospital in Cambridge. The Centre comprises the MRC Cancer Cell Unit and groups belonging to the University of Cambridge Department of Oncology.

Career Development Fellow

£25,368 - £31,048 pa (depending on experience)

Applicants are invited to apply for a post within the group of Dr Guillermo de la Cueva-Mendez. The laboratory's primary interest is in exploiting the prokaryotic toxin-antitoxin pair Kid/Kis in biotechnology and therapeutic strategies against cancer. In this context, you will undertake research to develop strategies aiming to exploit differences between cancer and normal cells to either a) Modulate the Kid-to-Kis ratio differentially in cancer cells and normal cells or, b) Allow therapeutic delivery of Kis and Kid genes (or their encoded proteins) to cancer cells in animal models.

You must have good interpersonal and communication skills and offer multi-disciplinary collaborations. You should send a full CV and a cover letter with your application, justifying how your skills and experience could contribute to any of the two projects offered above, and provide examples when appropriate (relevant literature can be found at http://www.hutchison-mrc.cam.ac.uk/de_la_Cueva_Mendez.html).

The appointment is a Career Development Fellowship designed to provide additional postdoctoral training in an excellent research environment, and is for 3 years on MRC salary band 4 (range £25,368 to £31,048 pa) with a minimum starting salary of £25,368 pa depending on the level of postdoctoral experience. Benefits include a flexible pay and reward policy, excellent holiday entitlement, MRC final salary pension scheme and access to excellent on-site leisure facilities.

Applications for this role must now be made online at http://jobs.mrc.ac.uk quoting reference **CCU08/092** please include a full CV with your application. If you do not have internet access or experience technical difficulties please call 01793 301260.

Closing date: 20th March 2008.

For further information about the MRC visit www.mrc.ac.uk

The Medical Research Council is an Equal Opportunities Employer and operates a strict no smoking policy.

'Leading science for better health'

U125819R

***icipe* – African Insect Science for Food and Health**

SCIENTIST – PROJECT CO-ORDINATOR/ IPM SPECIALIST

icipe – based in Nairobi Kenya is looking for a highly motivated scientist to fill a 3 year position as Coordinator of a project on Integrated Control of Thrips in vegetables in East Africa. The project seeks to develop environmentally friendly control options in vegetable production systems in Kenya & Uganda. The Scientist will coordinate research on thrips ecology and development, field testing of IPM strategies, provide technical backstopping to national programme Scientists and supervise Students in the project. For more information, visit http://www.icipe.org/jobs

Requirements

PHD in entomology or related field with at least 3 years postdoctoral experience, proven track record of R&D relating to IPM of vegetable pests preferably thrips in horticultural crops, demonstrated high quality publications, experience in fundraising and project management. A competitive international compensation package will be offered to the right candidate. More information – visit our Website. Deadline – 15th March, 2008 or until position is filled. *icipe* is an EOE.

RW125758R

Universität Bielefeld

The **Cluster of Excellence 'Cognitive Interaction Technology' (CITec)** at Bielefeld University invites applications for a

Junior Professorship (W1, tenure track option W2) in Active Sensing

beginning October 1st 2008. The position will be associated with the Faculty of Biology. The successful candidate will explore active sensing, for example visuotactile integration, active vision, and animal navigation. He/She will connect research on biological sensing principles with novel approaches to sensor design in technology. Quantitative behavioural and/or neuronal investigations based on an appropriate animal system should be combined with computational modelling.

The professorship will play a key role in the recently established CITec at Bielefeld University. The successful candidate is expected to cooperate intensively with other biologically and especially technically oriented groups in CITec. The main research focus of CITec is to understand how we can endow technical systems with the necessary cognitive abilities to support humans at a level of semantic interaction offering true flexibility by virtue of adaptivity. To achieve this goal, CITec will unite research groups from computer science, robotics, biology, biomechanics, cognitive psychology and linguistics. CITec is structured around four key areas, intelligent motion, attentive systems, situated communication as well as memory and learning (homepage: http://www.cit-ec.org/).

Candidates should have strong teaching capabilities at the undergraduate and graduate levels and will be expected to teach in the pertinent undergraduate and graduate programmes.

Candidates must have a university and a qualified PhD degree in a pertinent field, strong scientific achievements and an outstanding research record. We welcome applications from severely handicapped people. We particularly welcome applications from women. Given equal suitability, qualifications and professional achievement women will be given preference, unless particular circumstances pertaining to a male applicant predominate.

Applicants are asked to send their documents no later than **31 March 2008** to the **Dean's Office, Faculty of Biology, Bielefeld University, D-33501 Bielefeld, E-Mail: dekanat.biologie@uni-bielefeld.de.**
The following documents are requested in PDF format: curriculum vitae, publication list, brief statements of research and teaching experience and interests, documentation of successful third-party funding, names and addresses of four references.

W125798R

JOHN WAYNE CANCER INSTITUTE AT
ST. JOHN'S HEALTH CENTER IS SEEKING A

SENIOR MELANOMA RESEARCH PROGRAM DIRECTOR

John Wayne Cancer Institute at Saint John's Health Center is a cancer research institute dedicated to the understanding and curing of cancer in order to eliminate patient suffering worldwide. Our mission is accomplished through innovative clinical and laboratory research and the education of the next generation of surgical oncologists and scientists.

The John Wayne Cancer Institute in Santa Monica, California seeks an individual to oversee the administration of the Melanoma Research Program and to participate in melanoma research.

Position assists the Chief of the Melanoma Program in planning and developing new research programs including facilitating communications on scientific issues and coordinating interactions with research collaborators. In addition, individual will organize the administrative functions of the program. Requires application of scientific knowledge, tactful guidance, and competent management of operational functions with the goal of establishing worthwhile and well-run scientific programs and identification of new program opportunities.

Qualifications include a MD or PhD with background in research and administration of biomedical cancer research and clinical trials along with 5-10 years of increasing responsibility in the management of clinical and research institute and/or hospital administration.

We offer competitive compensation and benefits package, as well as the opportunity to truly impact the conduct and result of medical research. Qualified candidates may email resumes and salary history to: Donald L. Morton, MD, at John Wayne Cancer Institute, 2200 Santa Monica Blvd., Santa Monica, CA 90404, or email Sandra.barnes@stjohns.org and Mortond@jwci.org.

JWCI and SJHC is an Equal Opportunity Employer.

NW125284R

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Cell Biosciences™

Assay and Business Development Professionals
Cell Signaling and Biomarker Technology

We are seeking entrepreneurial, highly motivated scientists and business development professionals to join our management team. Cell Biosciences is a well funded Palo Alto, Ca. based developer of protein analysis systems. We have built extraordinary teams of experienced employees, highly respected academic collaborators and a well regarded consortium of international health care investors. Our largest shareholder is the Wellcome Trust.

The ideal candidates will have an understanding of cell signaling applications in the research and pharmaceutical markets and/or have experience in selling and servicing sophisticated instrumentation and reagents.

We are expanding our business and are seeking individuals in the following areas:

Sr. Product Management – Palo Alto, Ca. – This technically oriented individual will work closely with cell signaling labs to understand how our products are used to solve customer problems and fit in their work flow. S/he will integrate this customer knowledge into Cell Bio's assay development, engineering and applications groups to assure that our products are designed to meet customer needs.

Head of Assay Development – Palo Alto, Ca. – As a key member of the management team, this individual will be responsible for prioritizing and managing immunoassays developed for our instrument. The ideal candidate will be knowledgeable in protein phosphorylation and biomarker development.

East Coast Business Development – Eastern US – Building on our initial collaborations with UCSF and Stanford, this individual is responsible for closing sales and initiating demonstrations of our technology with translational medicine researchers in cancer, diabetes and systems biology at East Coast institutions and pharma companies.

UK/Europe Operations & Development - England - As our first international employee, you will be responsible for closing sales and setting up our international applications, collaborations and service operations for Europe and the UK.

These positions offer excellent compensation packages including participation in a stock incentive plan.

Please send your CV to: HR@cellbiosciences.com

We look forward to speaking with you!

NW125652R

nature methods

Locum Assistant Editor

Nature Methods seeks a Locum Assistant Editor to join their editorial team for a period of six months to cover a maternity leave. The journal publishes high quality papers that represent major methodological developments, likely to be influential in the life sciences. In the tradition of *Nature* journals, this selection relies on a thorough peer review process.

For more information about the journal, see our website (**http://www.nature.com/nmeth**).

Members of the editorial team evaluate manuscripts, oversee the peer review process, commission and edit secondary materials such as Reviews, and write short pieces and editorials for the journal. The new editor will join our team in the NYC office of the larger Nature Publishing Group.

Candidates should have a broad interest in science, excellent communication skills, and a willingness and ability to learn new fields. Applicants should have completed a Ph.D. in any of the areas covered in *Nature Methods*.

To apply, please submit a CV, and a cover letter explaining your interest in the position and your possible start date to Human Resources Department, Nature Publishing Group, e-mail: **admin@natureny.com**

Applications should arrive as soon as possible with a *close date of February 28, 2008.*

NPG is an EOE.

nature publishing group npg

IN123698R

Green Chemistry Golden Opportunity

The global demand for bioindustrial, pharmaceutical and chemical products has erupted at the same moment the world is realizing the acute need for green technology. Codexis and its innovative approach to biocatalysis is helping major energy, pharmaceutical and chemical manufacturing clients dramatically reduce time, cost, energy and environmental impact. Codexis: the right company, at the right time. And if you're a ridiculously talented scientist, the right place for you is **www.codexis.com**.
Right now.

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NW125708R

Neurologix, Inc
Neuro-science
Pre-clinical Specialist

General background in CNS diseases, experience in developing study protocols to assess mechanism of action, safety, efficacy of CNS therapeutics, demonstrate capability of executing protocol, good writing skills. 3-5 years GLP experience, specific experience in brain disorders a plus. Competitive, comprehensive salary and benefits package.

Contact
Email: csapan@neurologix.net.

NW125289RL

Neurologix, Inc.
AAV Process
Development Specialist

Strong background in neurology/molecular biology, demonstrate experience developing viral-based delivery systems for biological indications, some background in viral processing and scaling in a GMP environment. Minimum 5 years experience. Specific experience in brain disorders a plus. PhD req'd. Competitive, comprehensive salary and benefits package.

Contact
Email: csapan@neurologix.net.

NW125290RL

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RESEARCH FACULTY POSITIONS

Department of Ophthalmology and Visual Sciences
University of Illinois at Chicago

The Department of Ophthalmology and Visual Sciences at the University of Illinois at Chicago invites applications from outstanding research scientists and physician-scientists for tenured or tenure-track faculty positions as ASSOCIATE or FULL PROFESSOR

Applicants must hold a Ph.D., M.D., or equivalent and have a strong publication record in the field of fundamental mechanisms of vision processes, and/or translational/clinical topics in vision and ophthalmology. We welcome applicants whose work will complement the existing strengths (cornea, lens, retina, retinal degenerations and disorders, and glaucoma) and/or add new areas of expertise such as molecular genetics, gene therapy and ocular immunology.

It is expected that the individual recruited will have an established track record of research grant support from federal and/or national funding agencies, and be funded at the time of appointment to the faculty position. S/he will be encouraged to mentor pre-doctoral students and postdoctoral scientists. A generous startup package, competitive salary commensurate with experience, and independent laboratory space will be provided.

Qualified individuals should submit a curriculum vitae, a brief description of research interests, and the names, addresses (including e-mail), and phone numbers of three references to: Ms. Laurie Walker, Department of Ophthalmology, UIC, 1855 W. Taylor Street, MC 648, Chicago, IL 60612. Please submit materials electronically to: lawalker@uic.edu.

University of Illinois at Chicago is an Affirmative Action/Equal Opportunity Employer.

NW125738R

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www.mssm.edu

MOUNT SINAI SCHOOL OF MEDICINE

SYNTHETIC CHEMICAL BIOLOGY

The Department of Structural and Chemical Biology at Mount Sinai School of Medicine invites applications for two tenure-track faculty positions at the level of Assistant or Associate Professor in the area of synthetic chemical biology. We seek outstanding candidates with demonstrated excellence in synthetic chemistry and chemical biology approaches to the understanding of the molecular basis of disease and development. We are committed to developing innovative research in mechanisms and processes of cellular signaling and gene regulation, and structure based design for novel therapies.

The successful candidates are expected to establish dynamic, independently funded research programs and participate in graduate training at the interface of chemistry and biology. Candidates must hold a PhD or MD/PhD degree and have a strong record of research accomplishments.

We offer a salary commensurate with experience and a competitive benefits package. Please submit a curriculum vitae, a brief statement of research interests and future plans, copies of 2-3 publications and three letters of reference (sent independently) to: Chemical Biology Search Committee, Mount Sinai School of Medicine, 1425 Madison Avenue, Box 1677, New York, NY 10029. Email: CSCB.search@mssm.edu. EOE

NW125004R

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McGILL UNIVERSITY
Director, Meakins-Christie Laboratories

The Department of Medicine of McGill University and of the McGill University Health Centre (MUHC) is inviting applications for the position of Director of the Meakins-Christie Laboratories (www.meakins.mcgill.ca/meakins/) a major International centre for research in respiratory diseases. The Meakins-Christie Laboratories are supported by an endowment and have a history of producing outstanding research in the area of respiratory diseases. The MUHC (www.muhc.ca) is one of the largest academic health providers in Canada with new and visionary leadership dedicated to the expansion of clinical, research, and community services. McGill University (www.mcgill.ca) is an English-speaking institution located in Montreal, one of North America's most cosmopolitan cities.

The Director of the Meakins-Christie Laboratories is expected to have an international reputation in research related to respiratory diseases. The successful candidate will be forward-looking, able to forge collaborative scientific relationships, and possess a solid understanding of and commitment to translational research. Applicants holding an MD degree who wish to engage in clinical practice must be eligible for licensure in the province of Quebec.

Compensation will be commensurate with qualifications and experience. An attractive package will be provided to the successful candidate including start-up funds. The candidate will be eligible for a tenured or tenure-track position at the Full Professor level.

Please submit a curriculum vitae including a list of publications, an outline of current research interests, and the names, addresses and contact numbers of three references within 30 days of publication of this advertisement to:

Dr. Barry I. Posner
Chair, Meakins-Christie Search Committee
Polypeptide Hormones Research Lab
3640 University Street, Room W3-15
Montreal, Quebec, H3A 2B2
E-mail: barry.posner@mcgill.ca

McGill University is committed to equity in employment and diversity. It welcomes applications from indigenous peoples, visible minorities, ethnic minorities, persons with disabilities, women, persons of minority sexual orientations and gender identities and others who may contribute to further diversification. All qualified applicants are encouraged to apply; however, in accordance with Canadian immigration requirements, priority will be given to Canadian citizens and permanent residents of Canada.

NW125849R

早稲田大学 WASEDA UNIVERSITY

Waseda Institute for Advanced Study
Call for Researchers

Waseda University is working to establish a research framework that enables young researchers to demonstrate their capabilities to the full. Waseda Institute for Advanced Study is currently recruiting researchers as detailed below.

- Research Topics:
  Mathematics and Information; Chemistry and Energy; Batteries; Robotics and Machinery; Physics; Environment and Architecture; Life and Bio-science; Health and Sports Medical Science; integrated research of the above
  *Applications are also accepted for research related to other themes or academic fields.
- Period of appointment:
  Three years from date of recruitment
  In principle, from October 1, 2008 to September 30, 2011
- Appointment Status:
  Assistant Professor, Visiting Associate Professor (full-time), Visiting Lecturer (full-time), Visiting Research Associate

Applicants must have doctorate or equivalent, or have expectation of degree by October 1, 2008. Further details and application forms can be obtained from our website: **www.waseda.jp/wias/english**
Contact: **wias-info@list.waseda.jp**

To apply, applicants must fill in the registration form online on the website, and mail all the required documents to the following address:

Waseda Institute for Advanced Study
attention: Researcher Employment
1-6-1 Nishiwaseda, Shinjuku-ku,
Tokyo 169-8050, JAPAN

Closing Date:
April 8, 2008
5pm (Japan time)

www.waseda.jp/wias

JP124894R

Deputy Director
Help create our future

- *Leading biomedical research organisation*
- *Five-year, full-time position*

The Bionic Ear Institute is seeking to appoint a recognised leader in either Biomedical Engineering or Neuroscience.

Building on our extensive knowledge and experience with the Bionic Ear, our vision is to expand on our current base in Melbourne, Australia. We aim to create a significantly larger, world-class institute that combines the disciplines of biomedicine and engineering to create bionic devices that address the medical challenges associated with the auditory and other neural systems. Progress towards achieving this exciting vision is well under way.

The position of Deputy Director is a challenging opportunity to provide an outstanding contribution to the research, profile, leadership and operational activities of the Institute.

For a copy of the position description and the application process please visit www.bionicear.org/jobs

For further information and a discussion in the strictest confidence, please telephone Professor Rob Shepherd, Director +61 3 9667 7517 or Linda Peterson, Executive Officer +61 3 9667 7538.

Applications close: Wednesday 23 April 2008.

www.bionicear.org

The Bionic Ear Institute

JP124524R

Universität Karlsruhe (TH)
Forschungsuniversität · gegründet 1825

KIT Karlsruhe Institute of Technology

The Fakultät für Physik at the Universität Karlsruhe (TH) invites applications for the position of a

Professor (W3) of Applied Physics

(replacement of Prof. C. Klingshirn (C4))

The position at the Institute of Applied Physics is available within the interdisciplinary Center for Functional Nanostructures (CFN) supported by Deutsche Forschungsgemeinschaft (DFG), the State of Baden-Württemberg, and the German Excellence Initiative. The CFN comprises some 70 subprojects headed by researchers of the Karlsruhe Institute of Technology (KIT), composed of Universität Karlsruhe (TH) and Forschungszentrum Karlsruhe. The research focus of the successful candidate should be in the field of **Biophotonics**. Broadly speaking, Biophotonics is concerned with the interaction of light and biological systems. This includes but is not limited to, advanced optical microscopy, spectroscopy, and sensing of biological systems as well as their manipulation via advanced optical tweezers and/or optical nano-surgery.

It is expected that the candidate contributes to the CFN by complementing existing activities. Further possibilities for collaboration exist within the newly founded interdisciplinary Karlsruhe School of Optics & Photonic (KSOP).

The teaching duties include lecture courses in physics for students of physics and also of other natural sciences and engineering disciplines.

Universität Karlsruhe (TH) is determined to increase the number of female faculty members. Therefore, applications of qualified women are strongly encouraged. In addition, Universität Karlsruhe (TH) is an equal-opportunity employer and in case of candidates with equal qualifications and aptitude, preference will be given to handicapped applicants.

In case of a first-time appointment as a professor, the initial contract will be of limited duration. Exceptions are possible.

Applications with standard documentations, including a list of teaching and research experience as well as five selected reprints of own publications, should be sent by **March 28th, 2008** to: **Universität Karlsruhe (TH), Dekan der Fakultät für Physik, D-76128 Karlsruhe, Germany.**

W125437R

香 港 大 學
THE UNIVERSITY OF HONG KONG

Founded in 1911, The University of Hong Kong is committed to the highest international standards of excellence in teaching and research, and has been at the international forefront of academic scholarship for many years. Of a number of recent indicators of the University's performance, one is its ranking at 18 among the top 200 universities in the world by the UK's Times Higher Education Supplement. The University has a comprehensive range of study programmes and research disciplines, with 20,000 undergraduate and postgraduate students from 50 countries, and a complement of 1,200 academic members of staff, many of whom are internationally renowned.

Associate Professor/Assistant Professor in the Department of Anatomy (Ref.: RF-2007/2008-474)

Applications are invited for appointment as Associate Professor/Assistant Professor in the Department of Anatomy, from as soon as possible, on a three-year fixed-term basis and with consideration for tenure after satisfactory completion of a second fixed-term contract.

The Department offers M.B., B.S.; B.D.S., B.NURS. B.Chin.Med.; M. Med. Sci., M. Res.Med.; M.Phil. and Ph.D. programmes. There is a full-time academic establishment of 12 faculty members, 1 research assistant professor, 1 scientific officer, 2 research officers and 6 post-doctoral fellows. Excellent research support and facilities, including state-of-the-art cellular and molecular biological equipment, are available. More information about the Department can be obtained at http://www.hku.hk/anatomy.

Applicants should possess a medical and/or Ph.D. degree in the biological or biomedical sciences, post-doctoral experience, and a strong record in teaching and research, which should preferably be directed towards one of the four major areas of research conducted in the Department, i.e. cancer and cell biology, functional genomics, neuroscience and reproductive biology. The successful candidate would be expected to undertake teaching duties at the undergraduate and postgraduate levels as well as engage actively in high quality research.

Annual salaries will be in the following ranges (subject to review from time to time at the entire discretion of the University):

Associate Professor	:	HK$622,740 - 963,060
Assistant Professor	:	HK$474,600 - 733,440

(approximately US$1 = HK$7.8)

A highly competitive salary commensurate with qualifications and experience will be offered. The appointment will attract a contract-end gratuity and University contribution to a retirement benefits scheme, totalling up to 15% of basic salary, as well as leave and medical/dental benefits. Housing benefits will be provided as applicable.

Further particulars and application forms (272/302 amended) can be obtained at https://www.hku.hk/apptunit/; or from the Appointments Unit (Senior), Human Resource Section, Registry, The University of Hong Kong, Hong Kong (**fax**: (852) 2540 6735 or 2559 2058; **e-mail**: senrappt@hkucc.hku.hk). **Closes March 22, 2008. Candidates who are not contacted within 3 months of the closing date may consider their applications unsuccessful.**

The University is an equal opportunity employer and is committed to a No-Smoking Policy

JP125514R

Imperial College London

National Heart and Lung Institute
Faculty of Medicine

Flow Cytometry Co-ordinator

£28,820 - £35,800 per annum

Imperial College is ranked fifth in the top ten universities of the world, according to the 2007 Times Higher Education Supplement league tables.

Professor Michael Schneider's research group focuses on cardiac regeneration and repair encompassing diverse aspects of cardiac development and biology. Scientific questions are addressed with a multidisciplinary and collaborative approach. We wish to recruit a highly motivated self-starter who will aid the group and collaborators by operating and co-ordinating the use of two newly acquired high-end instruments for flow cytometry. You should have proven experience with flow cytometry, preferably in a stem cell context. You will perform analysis and sorting of cells as well as optimisation of procedures for the purification of cardiac progenitors and embryonic stem cells (murine and human). The work will include the evaluation of novel surface markers and the engineering of fluorescent reporter genes for the identification of specific subsets of cells. You should integrate with the group with a co-operative, enthusiastic and flexible approach.

To obtain an application form and further details go to http://www3.imperial.ac.uk/employment. Alternatively write to the Recruitment Assistant, Imperial College London, Faculty of Medicine, G02, Sir Alexander Fleming Building, South Kensington Campus, Exhibition Road, London, SW7 2AZ quoting reference number **RB06-08**.

If you have any further queries, please telephone 020 7594 1956 or email rb.recruitment@imperial.ac.uk

Closing date: 6 March 2008.

U125820RM

Valuing diversity and committed to equality of opportunity

John Innes Centre

Postdoctoral Researcher

Applications are invited for a one year Postdoctoral Researcher to develop Transcription Factor Decoys (TFDs) as potentially novel agents to combat infectious disease. TFDs are small stretches of DNA that control expression of bacterial genes by sequestering transcription factors that could otherwise turn on disease-causing genes. This project will design and test TFDs for their ability to restore susceptibility to extant antibiotics, restore the latter's efficacy, and inhibit bacterial growth by inactivating essential genes. This project will be carried out collaboratively with Procarta Biosystems Ltd (www.procartabio.com), which is developing TFDs for clinical application. Further details of this project can be obtained from Professor Mervyn Bibb (mervyn.bibb@bbsrc.ac.uk).

Candidates should have, or expect shortly to receive, a PhD in molecular biology. Experience in microbiology would be preferred. Good communication and organisational skills with the ability to design, plan and interpret experiments in an independent and innovative manner are essential.

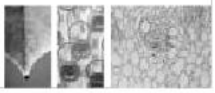
Salary on appointment will be within the range £24,200 to £27,200 per annum (pay award pending) depending on qualifications and experience.

For further information and details of how to apply, please visit our website http://jobs.jic.ac.uk or contact Human Resources, The Operations Centre, Norwich BioScience Institutes, Norwich, NR4 7UH, UK, 01603 450462 quoting reference 1001705

The closing date for applications will be 13th March 2008.

The John Innes Centre is a registered charity (No. 223852) grant-aided by the Biotechnology and Biological Sciences Research Council and is an Equal Opportunities Employer.

U125766R



Julius-Maximilians-
UNIVERSITÄT WÜRZBURG

BIOZENTRUM UNIVERSITÄT WÜRZBURG

The Faculty of Biology at the University of Würzburg, Germany, invites applications for a full-time faculty position

W3-Professorship (Chair) in Genetics and Neurobiology

(Succession to Prof. Dr. M. Heisenberg)

The Chair of Genetics and Neurobiology is integrated in the Biocenter at the University of Würzburg.

Responsibilities of the successful candidate include the organization and teaching of courses in Genetics and Neurobiology at the undergraduate and graduate levels. A prime research focus at the Biocenter investigates fundamental questions of nervous system function using arthropod model systems. Candidates for the position are expected to have an internationally established record of accomplishments in invertebrate brain research and should significantly contribute to this focus both in their research and teaching. Cooperation with the existing research programme in "Arthropod Behaviour" and with other research groups at the Biocenter, the Medical School, and the Departments of Psychology, Chemistry, Physics, and Informatics are encouraged.

Requirements: PhD, outstanding academic record including habilitation or equivalent academic qualifications, proven evidence of excellent teaching and research leadership, including successful track-record of securing external funding. Earliest starting date is October 1, 2008.

The University of Würzburg seeks to increase the number of female faculty and therefore especially encourages qualified woman to apply. Disabled applicants with equivalent qualification will be given priority.

Application Procedure:
Detailed instructions for applications and electronic submission can be found on our website (http://www.dekanat.biozentrum.uni-wuerzburg.de). Please submit applications as hardcopy and electonically **March 28, 2008** to:

Dekan der Fakultät für Biologie
Biozentrum, Am Hubland, 97074 Würzburg, Germany

W125449R

POST DOCTORAL RESEARCH SCIENTIST
PLANT SCIENCE DEPARTMENT

Applications are invited for a BBSRC-funded post-doctoral developmental biologist in the Crop Development and Genomics group of Graham King. The research project will investigate "modulation of seed size in oilseed rape *(Brassica napus)*" using a combination of developmental, genetic and cell-biological approaches. The project involves collaboration with Professor Rod Scott (University of Bath), with a research technician assigned to the project (based at Bath). The project will utilise knowledge of biological processes obtained in Arabidopsis to understand mechanisms controlling seed size in the related *Brassica* crop species.

Research in the King group focuses on molecular genetics and developmental biology of rapeseed composition and productivity by utilising comparative genomics and trait dissection in model and crop plants. This work is complemented by embryo and developmental studies in Arabidopsis and *Brassica*.

Candidates should have an excellent track record and experience in developmental biology (plant or animal) or genetics, and standard molecular techniques. Experience in embryology, cell biology, microscopy or micro-dissection would be advantageous.

We are searching for an experienced post-doctoral researcher who can work independently. Candidates should be highly motivated, creative and capable of contributing productively as part of a team. Proven ability to write scientific papers is desirable. Rothamsted Research has excellent laboratory and glasshouse facilities, along with first-class support in bio-informatics, statistics and bio-imaging. The Institute is renowned nationally and internationally for its leading-edge research and for its multidisciplinary expertise and emphasis on interaction across the full range of scientific disciplines.

The post is funded for 36 months at Band 6-PD. The appointment is full time with a starting salary normally in the region of £24,200 to £27,200 per annum (pay award pending). For informal discussion, please contact Dr. Smita Kurup (smita.kurup@bbsrc.ac.uk).

Previous applicants need not apply.

Apply by application form only, available with further particulars, from our website (http://www.rothamsted.bbsrc.ac.uk/careers/vacancies/Vacancies.html), or from the HR Group, Rothamsted Research, Harpenden, Herts, AL5 2JQ, rres.hr@bbsrc.ac.uk.

Please quote ref: 1020

Closing date: 10th March 2008

An Equal Opportunities Employer

U125781RM

ROTHAMSTED RESEARCH

Universität Stuttgart

Professorship (Chair, W3 mit Leitungsfunktion) for Technical Biochemistry at the Institute of Technical Biochemistry of Universität Stuttgart

(Current Chairholder Prof. R. D. Schmid)

(April 1st, 2009)

The Department of Chemistry invites applications for a full professorship for Technical Biochemistry. The candidate is responsible for teaching at both the undergraduate and graduate levels of "Technical Biochemistry" in the "Chemistry" and "Technical Biology" study programs. Research should be in the area of applied bio-catalysis with focus on the development of new bioproducts and new analytical methods for systems biology. This should support the research areas "Catalysis and Selectivity" and "Biochemical Technology" of the Department of Chemistry. Participation in the collaborative research center SFB 706 "Selective Catalytic Oxidation of C–H Bonds with Molecular Oxygen" and in the "Center of Systems Biology" of the Universität Stuttgart is expected and collaboration with other research groups in the fields of Biochemistry and Biotechnology is desired.

The requirements for employment listed in § 47 Baden-Württemberg university law apply; in case of first appointment as professor employment can be limited to three years.

To ensure full consideration of your application, all documents (CV, certificates, a short presentation of the scientific career, a list of publications with up to three reprints, a list of third-party funds and a short report on the present and future work) should be received by **31st March**, 2008. Please send your application to Prof. Dr. H.-J. Werner, Dekan der Fakultät Chemie, Universität Stuttgart, Pfaffenwaldring 55, D-70569 Stuttgart, Germany.

Universität Stuttgart wishes to increase the proportion of female academic staff and especially welcomes applications from women. Severely handicapped people will be given preference in case of equal qualification.

W125515R

University of Oxford

Department of Cardiovascular Medicine

Postdoctoral Research Fellow
John Radcliffe Hospital

£26,666 - £32,796 p.a.

Regulation of myocardial function and Ca^{2+}-handling by reactive oxygen species

We are looking for an experienced and highly motivated postdoctoral research fellow who would like to develop their scientific skills in a dynamic and interdisciplinary research environment. The focus of our research programme is to understand the role of nitric oxide and reactive oxygen species in the regulation of myocardial function in health and disease.

You would need to have first-hand experience in cardiac cellular electrophysiology techniques and/or confocal microscopy. You would need to be well-organised and able to interact with post-doctoral scientists and graduate students within our group and with collaborating groups in the Department. There are excellent opportunities for personal and career development within the group and in the Department.

The appointment is available from 1 May 2008 or as soon as possible thereafter. The project is funded by the Garfield Weston Trust for 3 years. Informal discussion regarding the position is encouraged – please contact yin-hua.zhang@cardiov.ox.ac.uk or barbara.casadei@cardiov.ox.ac.uk Further particulars, which detail the application procedure, should be obtained from http://www.admin.ox.ac.uk/fp/ The closing date for applications is 14 March 2008.

The University are Equal Opportunity Employers.
We positively encourage
applications from people of all backgrounds

U125807RIM

www.ox.ac.uk/jobs

Delphic

Treating you as an individual

Senior Scientist – Pharmacology (based in Liverpool)

Senior Scientist – Virology (based in Sittingbourne, Kent)

Senior Scientist – Molecular Genetics (based in Sittingbourne, Kent)

Salary Circa £45K plus bonus and 7.5% non-contributory pension

Delphic is a UK company based in central London with accredited laboratories on the University of Liverpool campus and on the Kent Science Park in Sittingbourne, Kent. Established in 2001, it is the UK's only specialist HIV diagnostics company, providing routine testing for clinics and specialist diagnostic and clinical trial management services for the pharmaceutical and biotechnology sectors. It is expanding into Europe, the US and South America and, while it has established its business and reputation in HIV, it is now increasing its infectious diseases portfolio and developing its 'individualised care' model for other therapy areas.

These three appointments are a crucial part of the next stage of the expansion of Delphic's business into new therapy areas and international clinical trials. Candidates will lead a team of scientific and technical staff in the development and expansion of rapid routine testing services in their specialist area, supporting both the existing clinical service and trials business and the development of new products and business opportunities across global markets.

Candidates must have a doctorate in biomedical sciences or life sciences with minimum post-doctoral experience of 5 years, preferably within the clinical trials, CRO, healthcare, pharmaceutical or diagnostics sectors. They must demonstrate a minimum of 3 years experience in laboratory management roles with budgetary and staff responsibilities, together with significant experience and knowledge of working within a laboratory QMS and of working with modern sequencing, automated technologies and information management systems. They must have a strong track record of achievement in developing and implementing new instrumentation, analytical methodologies, services and product lines.

For further information and a job description, please contact our Operations Manager, Nick Gardiner, on 020 7499 0777 or nick.gardiner@delphicdiagnostics.com, or send him your C.V. with a covering letter.

U125812R

Closing date for applications: 20 March 2008 DelphicDiagnostics

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THE UNIVERSITY of York

DEPARTMENT OF BIOLOGY

Postdoctoral Research Associate in Molecular Biology/Biochemistry

Ref: R0853

A position funded by the BBSRC is available for an enthusiastic and highly-motivated postdoctoral research associate to work on the molecular mechanisms and dynamics of DNA segregation machines in archaea. The aim of the project is to elucidate the function of archaeal factors in the process of genome segregation and to explore possible connections with cell division.

You should have extensive experience in DNA manipulation and protein overproduction, purification and analysis and will have a strong background in molecular biology, biochemistry or biophysics. You will join a young and expanding research team housed in a well-equipped laboratory in the recently constructed building of the Department of Biology, University of York.

Informal enquiries are encouraged and may be made to Dr Daniela Barillà (e-mail: db530@york.ac.uk).

The starting salary will be £26,666 per annum and this post is available for a period of up to three years.

For further particulars and details of how to apply, please see our website at: http://www.york.ac.uk/admin/persnl/jobs/ or write to HR Services, University of York, Heslington, York YO10 5DD, quoting reference number R0853.

Closing date for applications: 12.00 noon on Monday 10 March 2008.

The University of York is committed to diversity and has policies and developmental programmes in place to promote equality of opportunity.

U125759RIM

www.york.ac.uk

University of Glasgow

Department of Psychology

Research Assistant

£23,692 – £26,666

Applications are invited for a 21-month Postdoctoral Research Assistant position in a project entitled "Cognitive ERPs as translational endpoints and early clinical markers in Alzheimer's disease." This project is funded by the Translational Medicine Research Collaboration, a joint initiative supported by a consortium of Scottish universities, the Scottish Government, Scottish Enterprise, and Wyeth Pharmaceuticals. The project will use dense array (128 channel) EEG combined with cognitive probe tasks to investigate changes in brain and cognitive function that occur due to Alzheimer's disease. Specifically, the aim is to identify behavioural and electrophysiological biomarkers related to cognitive changes and treatment effects in Alzheimer's disease. The job requires preparing, carrying out, and analysing behavioural and EEG/ERP experiments with healthy volunteers and recently diagnosed Alzheimer's disease patients. A good background in statistics is necessary, and experience with Net Station, E-Prime, and Matlab are desirable.

Informal enquiries may be made to Kerry Kilborn (+44 (0) 0141 330 4686: k.kilborn@psy.gla.ac.uk).

For more information and an application pack please visit our website or contact Clare Alexander, Department of Psychology, University of Glasgow, Glasgow G12 8QQ. Tel: (+44 (0) 141 330 5090, Email: c.alexander@psy.gla.ac.uk

Applications comprising applicant information form, cv, covering letter, list of publications and names and contact details of two referees should be sent to Clare Alexander at the above address quoting Ref 14067/DPO/A3. Closing date: 5 March 2008.

The University is committed to equality of opportunity in employment.

www.glasgow.ac.uk Scottish University of the Year

U125768RIM

Imperial College London

National Heart and Lung Institute
Faculty of Medicine

Manager, Transgenic and ES Cell Core Facility

£28,820 - £35,800 per annum

Imperial College is ranked fifth in the top ten universities of the world, according to the 2007 Times Higher Education Supplement league tables.

Professor Schneider's research group focuses on cardiac regeneration and repair encompassing diverse aspects of cardiac development and biology. Scientific questions are addressed with a multidisciplinary and collaborative approach. We wish to recruit a highly motivated self-starter who will have primary responsibility for running and maintaining transgenic and ES services.

You should have proven experience in transgenic and ES technologies. You will be technically competent at a high level to ensure up to date technical support (such as DNA injection, ES cell injection, cell culture and aggregation techniques). You should integrate with the group with a co-operative, enthusiastic and flexible approach.

To obtain an application form and further details go to http://www3.imperial.ac.uk/employment. Alternatively write to the Recruitment Assistant, Imperial College London, Faculty of Medicine, G02, Sir Alexander Fleming Building, South Kensington Campus, Exhibition Road, London SW7 2AZ quoting reference number **RB05-08**.

If you have any further queries, please telephone 020 7594 1956 or email rb.recruitment@imperial.ac.uk

Closing date: 6 March 2008.

Valuing diversity and committed to equality of opportunity

U125817RM

nature materials

Associate Editor

Nature Materials is a prestigious international monthly journal (Impact Factor 19.194) covering all aspects of materials science and technology. We have an exciting opportunity available for a materials scientist or a chemist to join our editorial team as an Associate Editor working on all aspects of the journal.

We are particularly interested in applicants with expertise in physical chemistry and soft matter research but we would welcome applications from outstanding candidates in any area of materials science.

The ideal candidate should have a PhD and preferably postdoctoral experience with a strong research record. The successful candidate will play an important role in determining the representation of their field in the journal, and will work closely with the other editors on all aspects of the editorial process, including manuscript selection, commissioning and editing of Reviews and News & Views, and writing for the journal. A key aspect of the job is liaising with the scientific community through laboratory visits and international conferences.

This is a demanding and intellectually stimulating position. Broad scientific knowledge and training, excellent literary skills and a keen interest in the practice and communication of science are a prerequisite. The successful candidate must, therefore, be dynamic and outgoing and have excellent interpersonal skills. The salary and benefits will be competitive, reflecting the critical importance and responsibilities of this position.

The new editor will join our team in our London office. The *Nature Materials* team is part of a dynamic editorial and publishing environment that also includes *Nature*, *Nature Physics* and *Nature Nanotechnology*.

Applicants should send a CV (including their class of degree and a brief account of their research and other relevant experience), a News & View style piece (600 words or less) on a recent paper from related literature, and a brief cover letter explaining their interest in the post and their salary expectations.

The closing date for applications is Monday 25th February 2007.

To apply please send your CV and covering letter, quoting reference number NPG/LON/823 to Denise Pitter at londonrecruitment@macmillan.co.uk

All candidates must demonstrate the right to live and work in the UK to be considered for the vacancy.

nature publishing group npg

IN124059R

Naturejobs gives an explosive start to your career!

naturejobs

The Norwegian University of Science and Technology (NTNU) in Trondheim represents academic eminence in technology and the natural sciences as well as in other academic disciplines ranging from the social sciences, the arts, medicine, architecture to fine art. Cross-disciplinary cooperation results in innovative breakthroughs and creative solutions with far-reaching social and economic impact.

Faculty of Information Technology, Mathematics and Electrical Engineering

Department of Computer and Information Science

Professorship/qualifying fellowship

within the area Bioinformatics

A professorship or qualifying fellowship in Bioinformatics is available at the Department of Computer and Information Science, Faculty of Information Technology, Mathematics and Electrical Engineering, at the Norwegian University of Science and Technology (NTNU).

Further details about the Professorship can be obtained from Professor Kjell Bratbergsengen tel. + 47 73 59 34 39, cellphone + 47 906 17 185 or e-mail: kjell.bratbergsengen@idi.ntnu.no

Applications are to be sent to the Norwegian University of Science and Technology, Faculty of Information Technology, Mathematics and Electrical Engineering. The file number for the position IME 003-2008 is to be clearly stated on the application. **The application deadline is 2008-03-15.**

Complete announcement text on NTNU's website: http://nettopp.ntnu.no/?kat=N_JOBB

W124461R

NTNU
Norwegian University of Science and Technology

The University of Dublin | Trinity College
Ollscoil Átha Cliath | Coláiste na Tríonóide

sfi

SCHOOL OF GENETICS AND MICROBIOLOGY and SCIENCE FOUNDATION IRELAND

Professorship and Lectureship in Microbial Molecular Pathogenesis

The School of Genetics and Microbiology at Trinity College Dublin (http://www.genetics-microbiology.tcd.ie/) intends to appoint a Professor and a Lecturer in the area of Microbial Molecular Pathogenesis. These appointments are sponsored by the Stokes Professorship and Lectureship Programme of Science Foundation Ireland (http://www.sfi.ie/) to support the development of research in biotechnology in Ireland.

We seek exceptional candidates with international reputations capable of building competitive research teams in microbial molecular pathogenesis. For informal discussions of these posts please contact the Chair of Microbiology, Charles Dorman (cjdorman@tcd.ie) or the Head of School, Professor David J McConnell (david.mcconnell@tcd.ie).

Expressions of interest including a CV and the contact details of three academic referees should be sent to Professor Dorman by 31st March 2008.

Trinity College is an equal opportunities employer.

Professor Charles Dorman, Microbiology Department,
Moyne Institute of Preventive Medicine,
Trinity College Dublin 2, IRELAND

W125534R

Post Doctoral Fellowship

MRC National Institute for Medical Research

Situated in Mill Hill, North West London, NIMR is the largest MRC institute, supporting some 70 research groups and 500 bench scientists. The Institute provides excellent training for researchers in a multi-disciplinary environment and is equipped with state of the art facilities.
http://www.nimr.mrc.ac.uk/employment/

DIVISION OF IMMUNE CELL BIOLOGY

Reference: NIMR08/090

Regulation of NF-κB and ERK MAP kinase during immune responses

We are offering a 3 year MRC-funded Career Development Fellowship to work in a team investigating the regulation and function of ERK MAP kinase and NF-κB in innate and adaptive immune responses.

The project will involve analytical protein chemistry, molecular biology and a variety of immunological techniques, including primary cell culture, flow cytometry and in vitro / in vivo assays of primary immune cell function.

The ideal applicant should have experience in molecular immunology and / or signal transduction research.

For background information on the research area, see *Biochemical Journal*, **382**: 393-409, 2004; *Nature Immunology*, **7**: 606-615, 2006 and *Molecular and Cellular Biology*, **27**: 7355-7364, 2007

Further information is also available at **http://www.nimr.mrc.ac.uk/immcellbiol/ley**

For informal enquires, please contact **Steve Ley** (**sley@nimr.mrc.ac.uk**)

This is a training role supported by a comprehensive selection of courses and scientific learning which will equip the successful applicant for a career in research. Salary is from £26,808-£32,488 per annum inclusive of Location Allowance. MRC final salary Pension Scheme is available.

Applications for these roles must now be made online at **http://jobs.mrc.ac.uk**. If you do not have internet access or experience technical difficulties, please call 01793 301157.

The closing date is 20 March 2008.

The MRC is an Equal Opportunities Employer

U125672R

University of Veterinary Medicine, Vienna
The University of Veterinary Medicine Vienna (VUW), Austria, invites applications for the position of a

Full Professor of MICROSCOPIC ANATOMY AND EMBRYOLOGY

The area of assignment includes undergraduate lectures and courses in anatomy, histology and embryology for students at the VUW. Close collaboration with scientists in anatomy and pathology as well as clinicians and the core facility: tissue collection at the University are expected.

The Candidate must be a graduate of Veterinary Medicine or related fields and have a PhD, a Habilitation or an equal qualification in one of the areas: anatomy, histology, embryology with an excellent scientific track record. Ideally, he or she should be experienced in research and teaching and successful in competitive grant applications, and be ready to cooperative with other scientists both within and outside the VUW. The successful candidate is an outstanding, internationally recognised scientist with a strong interest in developing a research profile together with existing research foci at the VUW and has a commitment to excellence in teaching. Her or his scientific leadership will support the mentoring of students and junior faculty. The VUW seeks high potential scientific staff.

This is a tenure track position. Positive evaluation after five years will lead to a permanent position. Candidates from not German speaking countries are encouraged to apply. However it is expected – should they be appointed – that substantial knowledge in German is acquired within a reasonable time.

Application (if possible as hardcopy as well as on CD-ROM) can be submitted in German or English and require a

- letter of application
- curriculum vitae
- scientific track record including previous research and future research plans
- list of research grants
- list of the peer-reviewed publications with impact and citation factors (including reprints of five selected papers) and scientific lectures of the five last year.

University of Veterinary Medicine Vienna, c/o Winfriede Winkler,
Veterinaerplatz 1, A-1210 Wien, Austria
e-mail: winfriede.winkler@vu-wien.ac.at

The University of Veterinary Medicine Vienna is an Equal Opportunity Employer!

The closing date for applications is May, 15th 2008
http://www.vu-wien.ac.at/

W125298R

nature physics

Locum Associate Editor

Nature Physics seeks a Locum Associate Editor to join its editorial team for a period of nine months, to cover maternity leave.
Nature Physics is a prestigious journal covering all areas of research in physics. For more information about the journal, see our website (http://www.nature.com/nphys).
The ideal candidate will have completed a Ph.D. in a physics discipline, and postdoctoral experience is preferred (but not required). Key elements of the position include the selection of manuscripts for publication, as well as commissioning, editing and writing for the journal.
This is a demanding and intellectually stimulating role that calls for a keen interest in the practice and communication of science. The successful candidate will therefore be highly motivated and must possess excellent interpersonal skills.
The position will be based in our London office.
Applicants should send a CV (including a brief account of their research and other relevant experience); a research highlight in *Nature Physics* style (200 words or less) on a recent relevant paper in the literature; and a brief cover letter explaining their interest in the post and their salary expectations.
Applications should be sent to Denise Pitter, Personnel Assistant at londonrecruitment@macmillan.co.uk. Applicants should clearly mark on their submissions the reference number **NPG/LON/829**. Incomplete applications will not be considered.
All candidates must demonstrate the right to live and work in the UK to be considered for the vacancy.
Closing Date: Monday 31st March 2008

nature publishing group npg

IN125344R

The University of Edinburgh

The University of Edinburgh is an exciting, vibrant, research-led academic community offering opportunities to work with leading international academics whose visions are shaping tomorrow's world.

Lectureship or Senior Lectureship/Readership in Carbon Management

£33,779 – £40,335 or £42,791 – £48,161

A leading researcher/analyst in the field of Carbon Management, you will be based within the School of GeoSciences.

You must have proven capability as a researcher, as well as a commitment to the dissemination of knowledge through engagement with external organisations and media. You will pursue your own research as well as running an MSc programme on Carbon Management in collaboration with the School of Business and Economics.

Our preference is for appointment to commence in August, 2008.
Ref: 3008666NA.

Lectureship or Senior Lectureship/Readership in Experimental Geoscience & Earth Materials Science

£33,779 – £40,335 or £42,791 – £48,161

You will be a geoscientist with a strong research record focused on the synthesis, stability and properties of geo-materials under high pressure and temperature conditions, with proven laboratory skills and an exciting research agenda. Aspects of your research programme development will integrate activities between the School of Geosciences and the Centre for Science at Extreme Conditions at Edinburgh, and you will make a significant contribution to Undergraduate and Masters teaching and curriculum development of modern geo-materials within the School.
Ref: 3008667NA.

For both positions, the selection committee has the capacity to appoint at the rank of either Senior Lecturer or Reader (salary grade 9) to a suitably qualified candidate.

Apply online, view further particulars or browse more jobs at our website. Alternatively, telephone the recruitment line on 0131 650 2511. Closing date: 20 March 2008.

Committed to Equality and Diversity

www.jobs.ed.ac.uk

U125811R

The Department of Psychiatry and Psychotherapy at the **Faculty of Medicine** offers a

Professorship (W2-Professor) for Molecular Psychiatry

A fixed-term professorship with the status of a civil servant is available for a total of six years. The permanent appointment to professor following this term is possible in principle subject to the existence of the legal prerequisites.

The successful candidate will represent the discipline in research and teaching. The applicant should possess an own independent clinical orientated basic research profile in the area of molecular psychiatry. Modern cell- and molecular-biological research laboratories are at your disposal. Collaboration with the medical faculty research associates and a translation of research findings into clinical practice is expected. The Faculty of Medicine offers teaching programs in Medicine and Dentistry and in Molecular Medicine.

The candidate must have a university education in medicine or natural sciences and hold a M.D. or Ph.D. degree. Furthermore, excellent teaching skills, professional experience as a group-leader, high-ranking publications, and above-average research funds are required. These additional academic qualifications have been either obtained in a previous junior faculty position or during equivalent appointments outside the university.

Applicants must not be older than 52 years at the time of appointment. In urgent cases exceptions are possible but require the consent of the Bavarian Ministry of Science, Research, and Art and the Bavarian Ministry of Finances (Art. 10 Abs. 3 Satz 2 BayHSchPG).

The University of Erlangen-Nuremberg intends to increase the number of women in research and teaching positions and, therefore, strongly encourages female researchers to apply. Disabled applicants will be preferentially considered in case of equivalent qualification.

Please send a letter of application, a resume (picture required), a structured list of publications and teaching activities (one copy in written form, one copy on data CD) as well as officially certified copies of credentials and certificates to the office of the Dean of the **Medical Faculty of the University of Erlangen-Nuremberg**, Oestliche Stadtmauerstraße 30a, 91054 Erlangen, Germany. The deadline for application is **04th April 2008.**

Friedrich-Alexander-Universität Erlangen-Nürnberg

www.uni-erlangen.de

W125798R

The University of Edinburgh

The University of Edinburgh is an exciting, vibrant, research-led academic community offering opportunities to work with leading international academics whose visions are shaping tomorrow's world.

Postdoctoral Research Fellow

£27,466 – £32,796

You will contribute to a programme of high quality research investigating "The role of androgens in endometrial proliferation and differentiation". You will undertake research on the impact of androgen receptor function on endometrial gene expression including isolation of primary cells, cell culture, RNA and protein extraction, Western and qRTPCR analysis, preparation of samples for array analysis, validation of array data, cell based reporter assays, advanced imaging methods (confocal immunohistochemistry and live cell imaging).

You must also maintain accurate and up-to-date records and liaise with nurses regarding primary tissue collection. Presenting data at group meetings, section meetings, and meetings within the Institute will also be required. Along with regular reporting of progress to the group leaders and other members of the research team, you will disseminate results both by preparing text and figures for manuscripts as well as by giving presentations at national and international meetings.

This post is fixed-term for three years.

Apply online, view further particulars or browse more jobs at our website. Alternatively, telephone the recruitment line on 0131 650 2511. Ref: 3008671NA. Closing date: 6 March 2008.

Committed to Equality and Diversity

www.jobs.ed.ac.uk

U125810R

GEORG-AUGUST-UNIVERSITÄT GÖTTINGEN

The University of Göttingen invites applications for the positions of a

Professorship (W3 salary level, tenured) in „Biological Developmental Psychology“

and a

Professorship (W2 salary level, tenured) in „Biological Personality Psychology“

The positions will be available from October 01, 2008 onwards at the Georg-Elias-Müller-Institute of Psychology, belonging to the Faculty of Biology.

Teaching in **Biological Developmental Psychology** includes the whole field of developmental psychology as well as parts of psychological diagnostics. Applicants should have an excellent international publication record in cognitive and/or social developmental psychology as well as an interdisciplinary research profile linking psychology and biology (anthropology, primatology, behavioral biology, neuroscience).

Teaching in **Biological Personality Psychology** includes the whole field of personality psychology as well as parts of psychological diagnostics. Applicants should have an excellent international publication record in experimental personality psychology with a neuroscientific background. Their research profile should be distinguished by a neuroscientific approach to personality psychology.

Applicants for either of the two positions should be able to contribute to the newly founded Courant-Research-Centre “Evolution of Social Behaviour: Comparative Studies of Human and Non-Human Primates” (cf. http://www.uni-goettingen.de/de/58848.html), which is funded by the German “Excellence Initiative” and is jointly run by the Institute of Psychology, the Institute of Zoology and Anthropology, and the German Primate Center.

Applicants should hold a doctoral degree and have an outstanding teaching record. Appointments will be made by the university according to the laws of Lower Saxony (Niedersächsisches Hochschulgesetz Nds. GVBl. 5/2007, page 69). Further details are given on request.

Applications by scientists from foreign countries are explicitly encouraged. The University of Göttingen intends to increase the proportion of women in research and teaching, and hence strongly encourages female scientists to apply. Priority will be given to disabled persons with equivalent qualifications.

Applications with CV including scientific career, statement of teaching experience, overview of successful funding applications, list of publications and presentations, and a research plan (explaining the link to the above-mentioned Courant Research Centre) should reach the faculty no later than 6 weeks after the posting of the position and should be addressed to:

Dean of the Faculty of Biology, Georg-August Universität Göttingen
Prof. Dr. Stefan Schulz-Hardt, Untere Karspüle 1a, D 37073 Göttingen, Germany

All documents should be submitted on paper and as a single file on a CD. W125923R

The Baltic Summer School 2008

Basic and Clinical Aspects of Cardiac Arrhythmias

Organized by the Faculty Members of the Baltic Summer School,
coordinator: Prof. N-H Holstein-Rathlou, University of Copenhagen

Theoretical Course

17 – 29 August, 2008
University of Copenhagen, Denmark

Worldwide experts will present the current knowledge within the following fields:

- Cardiac ion channels and arrhythmias
- Intracellular calcium regulation in cardiomyocytes
- Advanced electrophysiology: models and prognostics
- Genetic approaches to human cardiac arrhythmias
- Ischemia and reperfusion: cardioprotection
- Bioinformatics and systems biology of the heart
- Gap junctions and intercellular communication in heart
- Cellular signalling in cardiac failure and hypertrophy
- Epidemiology and heart disease
- New preventive and therapeutic treatment strategies and targets

Laboratory Course

1-9 September, 2008
Universities of Copenhagen, Lund and Kiel

20 of the young scientists participating in the theoretical course will also have the opportunity to train in well-established research laboratories. The participants will join on-going research projects using state-of-the-art techniques ranging from genetic analysis of ion channels to epidemiologic research in cardiac disease.

Baltic Summer School Stipends

The Baltic Summer School is supported by the EU under the Marie Curie Program which has a special focus on young scientists with research experience at the late phase of their PhD studies as well as at the medical specialist or post-doc levels (or an equivalent academic training). Stipends are available under the program. For further information see the BSS homepage.
The meeting is also open for participants that do not receive a BSS-stipend.

Participation fee: 300 Euro
Application deadline: Friday, April 25, 2008
Further information: www.balticsummerschool.net
E-mail: bss@balticsummerschool.net

W125698E

Babraham Institute

Integrated Mechanisms of Cellular Identity and Homeostasis

Diamond Jubilee Conference
Babraham Institute, Cambridge UK
26th and 27th June 2008

Organisers: Anne Corcoran, Martin Turner, Raghu Padinjat
This meeting brings together international leaders in development and signalling to explore two convergent themes: Epigenetic mechanisms in development and Cellular responses to the environment.

Confirmed Speakers:

Yehudit Bergman, Israel
Wendy Bickmore, Edinburgh
Adrian Bird, Edinburgh
Dennis Bray, Cambridge
Doreen Cantrell, Dundee
Nal Divecha, Amsterdam
Julian Downward, London
Robert Feil, Montpellier
Amanda Fisher, London
Steve Jameson, Minnesota
Jeannie Lee, Harvard
Tom Misteli, NIH
Anjana Rao, Harvard
Kevan Shokat, UCSF
Austin Smith, Cambridge
Nicholas Spitzer, San Diego
Brigitta Stockinger, London
Azim Surani, Cambridge

Chairs: **Michael Berridge, Anne Ferguson-Smith, John Gurdon, Robin Irvine**

The conference will be held in the beautiful setting of The Babraham Institute, just outside Cambridge. Attendance is limited to 200 to facilitate maximum interaction between delegates. A limited number of student places will be available.

Early bird registration deadline is 31st March 2008.
Costs will be £150 for students and £250 for other academics.
Accommodation can be booked separately when registering. For more information and to register visit our website: http://www.babraham.ac.uk/DiamondConference/index.html

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2008 EVENTS

11-14 May 2008, Rome - CISAP3
3rd International Conference on Safety and Environment in Process Industry
www.aidic.it/CISAP3

8-11 June 2008, Naples - IBIC2008
1st Industrial Biotechnology International Conference
www.aidic.it/IBIC2008

6-8 July 2008, Rome - NOSE2008
International Conference on Environmental Odour Monitoring and Control
www.aidic.it/nose2008

9-11 November 2008, Naples - AAAS08
2nd Advanced Atmospheric Aerosol Symposium
www.aidic.it/aaas08

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Ever

Picture perfect.

Jeff Crook

It begins with a photo of a girl in a graveyard. She's clutching a tiny dog to her breast. She's dressed in rags and clothes she's made herself, knitting them together at night by the fire. She's leaning against a tomb as though she doesn't know who's buried in it. She's obviously been lured here by the photographer with the promise of a meal, a drink or a pipe of opium. The cemetery is merely backdrop. It may as well be a church or the gardens of Versailles.

He feeds the second photograph into the scanner. In this one, she leans slightly forward, displaying the plush white expanse of her Victorian bosom. A prostitute then, or perhaps a bone picker. It's impossible to tell her age: women of her class aged so quickly in those days, worn down by the impossibility of living. She has the eyes of a child orphaned by some hideous disaster.

She has the eyes of a woman sorely abused.

She has eyes the colour of a cold North Sea.

Her upper lip is cruel, insolently drawn by a pencil sharp as a pin. Her bottom lip is plump and childish as a baby's thumb.

The third photo is ravaged by fire. She pulls down her blouse to reveal the hint of a breast peeling from the charred uneven edge of the paper. Her face is utterly consumed except for the lobe of one ear. Her little dog licks its own nose. He hopes these will be enough, because they are the only three photos of her he has salvaged, and the machine needs at least three to regenerate her.

He leans back in his creaking chair and tosses the dregs of a warm gin martini past his beard, rises and retires to the kitchen to concoct a new one. Although he owns a Moonbeam Virtual Bartender with more than 10,000 recipes for perfect cocktails — a gift from a long-dead admirer — he prefers to mix his own, measuring out the ice, gin and vermouth by eye and instinct, peeling off a curl of lemon skin from a nearly naked lemon, and tasting that first exquisite sip while standing at the kitchen counter. By the time he returns to the living room, she's there.

Her little dogs wags its tail, yaps once, and leaps from her arms to the floor. It follows him back to the kitchen. He pours out some kibble into a bowl while its claws frantically slither and skid on the tiles. He sets the bowl on the floor for the famished little brute and returns to the living room where she still stands, having not yet moved or blinked or even breathed. He stands before her silently, admiring the shape and softness of her breasts, the round red apples of her cheeks, sipping his martini, unable to detect a single flaw.

JACEY

Finally he cups her dimpled chin in the palm of his hand and blows into her face, as you blow into the face of an infant to make it swallow. She blinks and turns her battleship grey eyes upon him for a furious moment, then grabs his proffered martini and sucks at it greedily, gulping and biting the glass, as though it is the first or perhaps last breath of life.

As she lowers the empty glass, she closes her eyes and begins to tremble all over. The glass slips from her hand and shatters on the floor, and in the kitchen her little dog gives a frightened yelp of pain. He steps close to her, slides his hand under her blouse and sucks her fleshy bottom lip into his mouth. She grabs his arm and wantonly pushes her pubic bone against his hip. Then she shudders and shoves him back with the muzzle of a Derringer that was hidden in her homemade bodice. "What the hell do you think you're doing?" she says.

"Kissing you," he says.

"You should be ashamed," she says. "You're old enough to be my grandfather."

"Oh, I'm far older than that," he says.

"If you want to do that, old man, you've got to pay me first," she says.

"How much?"

"Two dollars," she says.

"Two dollars?" he says. "I don't know. That's a lot of money. Let me see." He turns out his empty pockets. The buccinator, labial tractor and orbicularis oris muscles of her face contract, whetting the stilettos of her already dangerous upper lip, but compressing her lower lip into a luscious pout.

"I'm no whore," she says.

"Of course not," he says. "But you're bound to be hungry."

"Hungry?" she says.

"Yes. Would you like something to eat?"

"Not really," she says.

"No?" he sighs. "Oh dear. That's not good."

"What's not good?"

He's seen it happen before. Without a set of three complete photos, flaws are introduced into the machine's genitive modelling. The flaws present as a lack of some basic human need — warmth, companionship, self-preservation, procreation, water: for her it is food. Unless he forces her to eat, she will eventually starve to death.

"What are you doing?" she says as he reaches for the machine.

"I'm very sorry," he says.

"No wait. Please don't," she says. But he shakes his shaggy grey head sorrowfully. The machine spits out her photos. They flutter like brown autumn leaves to the floor.

"What just happened?" she asks.

"I'll be damned," he says in admiration. He's been so lonely for so long. He takes her hand and feels its tender strength. "You're a survivor." Maybe he's been wrong all along to seek perfection. ■

Jeff Crook, fantasy novelist and designer of 'Southern Gothic' apparel, lives in Memphis, Tennessee.

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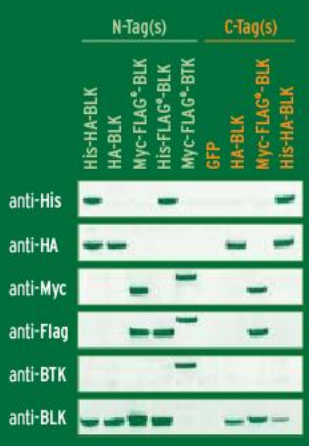

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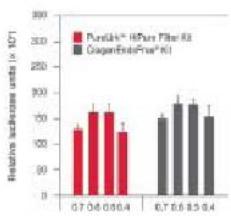

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